IN VIVO MONITORING OF AMINE-CONTAINING NEUROTRANSMITTERS BY CAPILLARY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
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To my dear mom.

ACKNOWLEDGMENTS

In everything I give God thanks. I would like to thank my advisor, Robert Kennedy, for his support, and members of the Kennedy group, past and present for their encouragement, support and friendship. A special thank you goes to my family (especially my wonderful mom) and friends who supported me through thick and thin and were always there to give a listening ear. I thank them all.

TABLE OF CONTENTS

page

Abstract of Dissertation Presented to the Graduate School
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There are many signaling molecules in the brain including L-amino acids, peptides, indoleamines, catecholamines and other small molecules. In most cases, samples generated *in vivo*, from microdialysis, sampling probes, or tissue extracts are analyzed for neurotransmitters by HPLC with electrochemical detection, radioimmunoassay, fluorometric detection, microelectrode, or fiber optic sensors. However, these traditional approaches are usually only capable of monitoring single neurotransmitters or a single class of neurotransmitters in a given experiment. A separation technique following sampling is attractive because it permits the simultaneous analysis of multiple analytes. Simultaneous monitoring of neurotransmitters is important to investigate the interactions and interconnections among different neurotransmitter systems, to correlate behavior, stimuli or drugs to neurotransmitter activity and to determine the role of each neurotransmitter.

The increased interest in stereochemical aspects of pharmacological activity and drug design has led to the development of new, specific and sensitive methods for the determination of enantiomers. Recent studies have provided evidence indicating that D-amino acids, such as D-serine (Ser), D-aspartate (Asp) and D-alanine (Ala), present in mammals may have important functions. Thus, it has become important to be able to determine the D and L forms of amino acids in biological or physiological samples.

In this work, a sensitive and quantitative method for the simultaneous detection of various amine-containing neurotransmitters representing different classes was developed. The method is based on pre-column derivatization with o-pthaldialdehyde (OPA) and a thiol, gradient elution; on-column preconcentration (50 μm i.d. capillary columns) and detection by amperometry with a carbon fiber microelectrode. With this approach, simultaneous separation of L-amino acids, catechol and indole amines with concentration detection limits of less than 1 nM was achieved following injection of 250 nL samples. This represents the first example in which different classes of neurotransmitters are monitored simultaneously without the use of sample splitting. The method was extended to include amino acid enantiomers. Samples (100 nL) containing amino acid enantiomers, non-chiral amino acids, catechol and indole amines resulted in detection limits of 3- 280 nM. There are no known reports of simultaneous analysis of L- and D-amino acids by amperometry following derivatization with OPA and a thiol.

CHAPTER 1
INTRODUCTION

Neurotransmitter Monitoring

The brain is composed of neurons, which communicate with each other by releasing chemical messengers (neurotransmitters) at junctions called synapses. Over the past several years, the role of amine-containing neurotransmitters in the central nervous system (CNS) has received considerable attention. There are at least 50 – 100 different neurotransmitter varieties in the brain, each identified with a specific behavior or function. Although the dynamics of neurotransmitter activity in the brain is not fully understood, it has been hypothesized that abnormalities in their regulation and secretion may have far reaching implications for both pathophysiology and treatment of associated diseases (Magnusson et al., 1992). Pathological roles of neurotransmitters include neurological diseases such as Parkinson's, Huntington's, Alzheimer's and epilepsy (Greenamyre and Young, 1992; Carlsson and Carlsson, 1990; Meldrum, 1995). Neurotransmitter functions are implicated in learning disabilities, feelings of hunger, mood disorders and schizophrenia (Kalra et al., 1999; Magnusson et al., 1992).

In previous studies, neurotransmitter monitoring *in vivo* has been evaluated by the use of implantable sensors, microelectrodes, but they cannot be reliably used *in vivo* because of difficulties encountered in attempts at calibration (Wightman and Zimmerman, 1990; Mitchell et al., 1994). However, the progress in the study of neurotransmitter dynamics has been hampered by the lack of a simple method capable of monitoring multiple neurotransmitters and their metabolites in biological fluids with high sensitivity and

selectivity. A separation technique following *in vivo* sampling is attractive because it permits the simultaneous analysis of multiple neurotransmitters (Wightman et al., 1991; Hu et al., 1994). Simultaneous monitoring of neurotransmitters is important when studying interactions and interconnections among different neurotransmitter systems. Quantitative measurement helps to correlate behavior, stimuli or other pharmacological effects to neurotransmitter activity in the brain.

Neurotransmitter Categories

Neurotransmitters are believed to undergo a cycle involving synthesis and packaging into vesicles in the pre-synaptic cell and release into the synapse through exocytosis after fusion of the vesicles with the plasma membrane in response to elevated calcium levels followed by rapid removal and/or degradation (Snyder and Kim, 2000). However, the criteria for defining a neurotransmitter remain a difficult undertaking. Substances not meeting all the criteria of a neurotransmitter are referred to as 'putative' neurotransmitters or 'neuromodulators'.

Neurotransmitters can be classified into two broad categories based on their size: small molecule neurotransmitters and neuropeptides. Shown in Figure 1-1 are the structures of the most studied neurotransmitters. Small molecule neurotransmitters include acetylcholine (the first to be discovered), amino acids, monoamines, purines and gases which mediate rapid synaptic actions. Aspartate (Asp) and glutamate (Glu) are the major excitatory amino acid neurotransmitters responsible for normal synaptic neurotransmission, glycine (Gly), γ-aminobutyric acid (GABA), taurine (Tau) the major inhibitory neurotransmitter. Other amine neurotransmitters are metabolically linked to amino acids, for example, tyrosine is converted to dopamine (DA), tryptophan (Trp) to

serotonin (5-HT). As such monoamines such as catecholamines and serotonin (5-HT) are also found to act as neurotransmitters in the CNS. Present in the brain are other small amine-containing signaling molecules. Examples classified into groups based on function are given in Table 1-1.

Table 1-1 Examples of the amino acid molecules in the brain

Transmitters	L-Glu, GABA
NMDA receptor site modulators	Gly, D-Ser
Neuroactive amino acids	L-Gln, o-Pea
Non-Neuroactive amino acids	L-Ser, L-Ala

Gln represents glutamine, o-Pea, o-phosphoethanolamine.

Neuropeptides are large molecules composed of 3- 36 amino acid residues that modulate slower on-going synaptic functions. Some examples include endorphins (enkephalins) involved in pain reduction, pleasure and hibernation, substance P, which mediates the experience of pain, and neuropeptide Y, a neurotransmitter linked with human eating disorders (Kaye, 1990).

Derivatization of Amines

The determination of amines including amino acids and mono amines in a complex mixture is difficult because the compounds are usually present at very low concentrations. Amines are not readily measured by high performance liquid chromatography (HPLC) because of their high polarity and low response if any to ultraviolet (UV), visible, fluorescence and electrochemical detectors. Neuroactive amines, such as dopamine and serotonin, are naturally electroactive; electrochemical detection has been used with capillary electrophoresis (CE) to detect these compounds in

neurons (Olefirowicz and Ewing, 1991; Kristensen et al., 1994) and human lymphocytes (Bergquist et al., 1991). However, there are many other neuroactive substances present in biological samples which are not natively electroactive. Chemical derivatization can be used to label these compounds with an electroactive or fluorescent tag. Derivatization involves the use of chemical reagents and reactions to convert the non-detector responding analyte into one or more derivatives that have enhanced chromatographic and/or detector properties. Labeling of amino acids by derivatization reactions results in increased sensitivity and methods of detection. Traditionally, ion-exchange chromatography and detection following post column derivatization have been used for the quantitation of amines in various samples (Spackman et al., 1958). This method has several drawbacks including requirement for specialized apparatus, long analysis time (1-3 hr) and post column derivatization, which makes the limit of detection susceptible to elution buffer contaminants. In post column derivatization, the reaction does not have to yield a single, stable product, provided that the derivatization reactions are reproducible. There are several disadvantages associated with the use of this approach: excess reagents often interfere with detection, additional pumps are required for a non-pulsating supply of reagents, reaction solvents must be miscible with the mobile phase used for separation and efficient mixing of the derivatization reagents with the column effluent is required. Post-column derivatization requires an on-line reactor, which causes peak broadening due to dilution and mixing, makes the system more complex and causes additional baseline noise. Pre-column derivatization has circumvented some of these problems. In addition to increasing the detectability, pre-column derivatization may also improve the selectivity by changing the polarity of the individual amino acids, thereby allowing for alternative

separation techniques. However, the excess reagents present in the mixture must be chemical or physically removed from the sample solution prior to injection or must be resolved from the peaks of interest.

A wide variety of derivatization reagents for amino compounds have been employed in the high sensitivity and selectivity methods of LC and CE (Toko'oka, 1999; Bardelmeijer et al., 1998). Labeling techniques using o-phthaldialdehyde (OPA) (Roth, 1971; Lada and Kennedy, 1996), naphthalenedialdehyde (NDA) (Zhou et al., 1995), 3-(4-carboxybenzoyl)-2-quinoline carboxyaldehyde (CBQCA) (Liu et al., 1991) and fluorescein isothiocyanate (Hernandez et al., 1993) have also been used in conjunction with HPLC or CE followed by electrochemical or laser induced fluorescence (LIF) detection. Amino acids have been analyzed by HPLC by forming 5-dimethylamino-naphthalenesulfonyl (dansyl) or phenyl-thiohyantoin (PTH) derivatives that can be separated on reversed phase columns and detected at low concentrations (Bayer et al., 1976; Elion et al., 1978). Dansyl derivatives require the use of 2 columns for HPLC analysis; the excess reagents and side products cause serious interferences at the sensitivity level needed for most applications (Bayer et al., 1976) and the lengthy preparation required for PTH derivatives limit their usefulness for routine amino acid analysis (Edman and Henschen, 1975). Another reagent, fluorescamine, reacts rapidly (< 1s) and quantitatively with primary amines, however the reaction products are bulky and for each amine derivatized, 2 fluorescent products are formed (McHugh et al., 1976).

OPA derivatives

Roth first demonstrated that OPA reacts with amino acids at high pH in the presence of 2-mercaptoethanol (2-ME) in aqueous alkaline solution to produce

fluorescent compounds (Roth, 1971b). The mechanism of this reaction and fluorescence properties of the products formed in reaction of OPA with various thiols have been studied and the fluorescent product identified as a 1-(alkylthio)-2-alkylisoindole, (Simmons and Johnson, 1976). Shown in Figure 1-2 is the reaction of OPA and a primary amine with tert-butyl thiol. OPA- amino acid derivatives are known to contain an isoindole group (Lindroth and Mopper, 1979). Likewise tryptophan, an indole is a naturally electroactive amino (most amino acids, with the exception of tryptophan (Trp), tyrosine (Tyr) and cysteine (Cys) are not naturally electroactive (Bennett et al., 1981)). In light of this Joseph and Davies demonstrated the fluoremetric and electrochemical detection, Figure 1-3 in series of amino acids following derivatization with OPA and thiol (mercaptoethanol) prior to reversed phase gradient elution (Joseph and Davies, 1983). However, the electrochemical properties of OPA derivatives have not been widely explored. Amino acid analysis can be performed by liquid chromatography with electrochemical (LC-EC) or laser-induced fluorescence detection (LIFD) following derivatization of the amino group (Cheng et al., 1992; Keating et al., 1993; Cooper et al., 1994; Jensen and Marley, 1995). The majority of reagents used in derivatization are organic thiols; thus the preferred mode of separation is reversed-phase chromatography. The LC system used for separation depends on the number and targeted amino acid acids. For example, analysis of several amino acids is performed by gradient elution (since the derivatives have a wide range of hydrophobicities). Selective analysis or analysis of few amino acids can be accomplished by isocratic elution. This is the principle used to separate physiological amino acids (L –form). There is increased interest in stereochemical aspects of pharmacological activity and drug design, therefore the drive to

separate enantiomers. Chiral thiols can be used in conjunction with OPA and chiral amino acids to form diastereomers that can be separated using conventional reversed phase chromatography. The reaction of Boc-L-Cys with OPA to form a pair of diastereomers (L-L and L-D form) is shown in Figure 1-4.

A significant problem with the pre-column derivatization approach is the variable stability of the substituted isoindoles obtained with some amino acids (Allison et al., 1984; Hogan et al., 1982; Lindroth and Mopper, 1979). For L-amino acid analysis, the variable stability of OPA/β-ME derivatives makes the method insufficient for adequate analytical precision (Chen et al., 1979; Buteau et al., 1981; Lee and Drescher, 1978; Larsen et al., 1980). The amount of instability shown by the OPA derivative is dependent on the thiol used, the primary amine under investigation, and the solvent (Chen et al., 1979). Selection of a suitable thiol provides a means of enhancing derivative stability. Upon substitution with a bulky thiol, such as tert-butyl thiol, more stable derivatives are formed. For example, the GABA derivative formed from 2-mercaptoethanol or ethanethiol has a half life of only 8 minutes, however the use of a more bulky thiol, tert-butyl thiol (t-BuSH) results in improved stability ($t_{1/2}$ 410 minutes) (Allison et al., 1984, Smolders et al., 1995). Replacement with t-BuSH results in more stable derivatives, allowing for sensitive determination of amino acids in complex matrices without concerns regarding degradative loss (Allison et al., 1984; Simmons and Johnson, 1977). In addition, an approximately two-fold increase in electrochemical response was observed compared to a decrease in fluorescence efficiency (Allison et al., 1984),

Separation of OPA derivatives can be carried out using C-8 or C18 columns (Hodgin, 1979; Lindroth and Mopper, 1979). The influence of the length of the bonded alkyl chain

on the separation of an amino acid mixture using identical gradients was investigated, when C-8 columns were used, retention times were reduced by 25% compared to C-18 columns. In another report, it was observed that C-8 columns showed less peak shape degradation, peak tailing and loss of peak intensity. Also re-equilibration times were less compared to the C-18 columns (Cooper et al., 1986).

Microdialysis Sampling

Microdialysis sampling has become a standard procedure that enables the investigation of biochemical events in the extracellular fluid of tissues, organs or biological fluid (Ungerstedt, 1991). Microdialysis involves the use of a probe (illustrated in Figure 1-5) constructed with a membrane of known molecular weight cut-off which is implanted directly into the tissue of interest and perfused with artificial cerebral spinal fluid (aCSF). The size exclusion nature of microdialysis probes results in protein-free samples which can be directly assayed without the need for further sample clean-up. Microdialysis sampling reflects the extracellular fluid concentration and historically, is used to correlate the relationship between the activity of neurotransmitter as a function of physiological or pharmacological events (Justice, 1993). There is some controversy concerning the ability to establish the extracellular concentration of neurotransmitter or the *in vivo* recovery of a microdialysis probe. As a result, when comparing a series of drugs or other treatment in a given brain region, the data is generally expressed as a percent of baseline, i.e., a reference point.

Analysis of small molecule neurotransmitters in dialysate must be very sensitive because they contain sub-picogram (low nanomolar) amounts in only a few microliters of perfusates. Fractions obtained *in vivo* from microdialysis can be assayed by several techniques, all with the potential to profile multiple analytes in each case. The methods of

assay available are complicated, tedious, and insensitive or require expensive instruments. Free amino acids are separated on ion-exchange columns, followed by post column derivatization with ninhydrin (Moore et al., 1958) or OPA (Benson and Hare, 1979). Labeling techniques using OPA (Lada and Kennedy, 1996; Roth, 1971), NDA (Zhou et al., 1995), CBQCA (Liu et al., 1991), and fluorescein isothiocyanate (Hernandez et al., 1993) have also been used in conjunction with HPLC or CE followed by EC or LIF detection. These methods have been used to analyze different classes of primary amines; however, analysis has been limited to the quantification of a few analytes in each experiment and often requires a long analysis time.

HPLC- Basic Principles

HPLC is an analytical tool in which the separation of solute molecules is achieved based on their differential retention by a stationary phase (SP). A chromatogram is an elution profile for pure compounds represented as individual peaks in which the concentration of solutes eluting from the column, based on detector response, is plotted against time.

After injection into a HPLC column any solute molecules that do not interact with the SP is eluted at time t_o in the void volume v_o. The void volume represents the volume (space) occupied by the pores in the particles and the volume (space) between the particles of the stationary phase. The retention time t_R is the time required to pass, following injection, from one end through to the other end of the column and into the detector. Resolution, R_S, is a measure of how well a given HPLC system separates two components in a mixture.

R_S = difference in retention time/ average peak base width

$$= 2(t_{R\,(B)} - t_{R\,(A)} / (W_A + W_B).$$

Resolution can also be described in terms of the selectivity factor (α), retention factor (k') and column efficiency factor (N), i.e.,

$$R_S = \alpha k' N.$$

Resolved compounds elute in different solvent volumes (V_R) because of differential retention. The retention factor (k') is a measure of the degree of retention and can be calculated from the following equation,

$$k' = (V_R - V_O) / V_O = (t_R - t_O) / t_O$$

where k' is the number of column volumes required to elute a particular solute, V_O and t_O represent the void volume and void time respectively. A related concept is selectivity, defined as the relative separation between adjacent solute peaks, or simply the ratio of the retention factors for two peaks, α.

$$\alpha = k'_1/k'_2$$

The number of theoretical plates (N) is a measure of column performance or efficiency and can be calculated from any peak in a given separation. In general, the more theoretical plates, the better the column.

$$N = 16(t_R/W)^2$$

where W is the peak width at baseline. However the peak width at half height has been found to be more useful for peaks not completely resolved, peaks that exhibit tailing, or asymmetrical peaks. Alternately,

$$N = 5.54\ (tR/w^{1/2})^2$$

where $w^{1/2}$ is peak width at half height. Since N is independent of retention time but proportional to column length, height equivalent to theoretical plate (HETP) is a better

measure of column efficiency because it allows for comparison between columns of different lengths.

$$H = L/N$$

where L is the length of the column in mm and H the plate height. The reduced plate height, h, is equal to H/d_p where d_p is the particle diameter. The calculation of column efficiency as a function of experimental variables (column length, column diameter, mobile phase flow rate, temperature, sample molecular weight) can be summarized by the Knox Equation,

$$h = Av^{1/3} + B/v + Cv$$

where $v = ud_p/D_m$, v is the reduced mobile phase velocity, u the mobile phase velocity and D_m the solute diffusion coefficient. For the above parameters R_S values greater than 1.5 indicate complete separation. Optimum values of k, α, N are 2-10, 1.15 and 20,000 respectively. For a well-packed column with 5 µm particles, H values usually range from 0.01 – 0.03 mm (Snyder et al., 1983).

Reversed Phase Chromatography (RPC)

Reversed phase chromatography is the most widely used HPLC technique. The majority of stationary phases used in RPC are silica-based with covalently bonded alkyl chains of different lengths, which provides a hydrophobic surface to facilitate separation. As the alkyl chain length increases, the strength of hydrophobic interaction increases. The mobile phase involves the use of an organic solvent which facilitates sample concentration at the head of the column provided the initial organic solvent strength is low enough. The sample components and mobile phase compete for sites on the silica support; retention is therefore based on the differential hydrophobic interactions of

sample components with hydrophobic groups on the bonded phase. In this way, polar or weakly hydrophobic solutes elute quickly. The solutes adsorb onto the hydrophobic surface and remain bound until a high concentration of organic solvent displaces it from the solid support. In general, the elution of solutes is sensitive to minute changes in organic solvent strengths. As a result, almost all RPC methods are carried out using gradient elution.

Linear Solvent Strength (LSS) Gradient Elution

All solutes are initially retained at the column inlet, however, the mobile phase strength can be linearly increased during the separation until the k' values become small enough to allow migration along the column. For solutes eluting at different times, LSS gradients provide roughly equal values for k', resulting in $\overline{k}$, the average capacity factor for the separation. The average capacity is related to the gradient profile by the gradient steepness parameter b,

$$\text{where } \overline{k} = 1/1.15b.$$

Alternately, $\overline{k}$ can be expressed as a function of gradient time (t_G), flow rate (F) and column dead volume (V_m),

$$b = \Delta\Phi S V_o / t_G F,$$

where $\Delta\Phi$ is the change in organic modifier during the gradient, S is the solvent strength of the modifier, V_o the void volume of the column and t_G the time over which the gradient is linearly changed. The value of b should be in the range 0.43 – 0.087 for the majority of applications (Snyder et al., 1979).

Table 1-2 Solvent strength of various organic modifiers

Methanol	Acetonitrile	Ethanol	Acetone	Dioxane	2-propanol	THF
3.0	3.1	3.6	3.4	3.5	4.2	4.4

Miniaturization

Microcolumn Separation

Traditionally, most of the *in vivo* samples derived from brain regions were obtained from tissue and fluid samples that contain high levels of neurotransmitters and their derivatives (Magnusson et al., 1992). The majority of samples analyzed today are perfusates from microdialysis sampling in which the neurotransmitter levels are low. The small sample size and low concentrations obtained from microdialysis experiments creates new analytical challenges, thereby increasing the demands on sensitivity. Fraction collection of perfusates coupled with HPLC and radioimmunoassay (RIA) has been used to determine the extracellular concentration of some neurotransmitters. HPLC, while capable of separating multiple neurotransmitters with good resolution, requires long analysis times and suffers from a lack of mass sensitivity. RIA offers the advantage of high selectivity but is used to monitor a single analyte in each experiment and requires the use of large sample volumes. This makes them unsuitable for the analysis of multiple analytes contained in small fractions.

Miniaturized separation techniques such as packed capillary columns (inner diameters < 300 μm) and capillary electrophoresis have received considerable attention over the last few years (Kennedy and Jorgenson, 1989; Jorgenson and Lukacs, 1983). Microcolumn separations are promising methods of quantitation that can provide maximum selectivity

and selectivity for the analysis of small and limited samples obtained *in vivo* following microdialysis sampling. In particular, capillary chromatography is attractive due to its high mass sensitivity, minimum mobile phase requirement and ability to separate, with high resolution, multiple compounds following a single injection. Today, microcolumn separations are essential techniques in sample-constricted applications such as bioanalysis, proteomics, high-throughput screening and combinatorial chemistry.

The advantages offered due to miniaturization can only be exploited when the same sample size (volume) can be loaded and the operating conditions of the columns are identical. The most important advantage of capillary chromatography is the increase in mass sensitivity associated with its use. For example, the use of a 1 mm i.d. column should result in a mass sensitivity increase of about 20 fold compared to the conventional 4.6 mm i.d. column when operating at the same linear velocities. Table 1-3 illustrates the theoretical gains in mass sensitivity for columns of different internal diameters.

Of the three main types of capillary liquid chromatography, fully packed capillaries are the most common. Advantages of capillary liquid chromatography over conventional liquid chromatography include higher detection sensitivity for a given amount of sample (mass sensitivity enhancement is illustrated in Figure 1-6 in which the same sample is injected onto a capillary and conventional column), higher column efficiencies and sub-microliter flow rates which are ideal for direct coupling with electrospray ionization (ESI)-MS or nuclear magnetic resonance (NMR). In addition, capillary LC separations are ideal when sample volumes are small and limited such as in biological systems (single cell analysis, single vesicle analysis). Downscaling of

conventional HPLC to capillary LC is possible with appropriate flow splitters, micro-connectors, tubing and fittings (Vissers, 1999).

Table 1-3 Theoretical gains in mass sensitivity for different columns.

Column ID (mm)	Cross-sectional area (cm^2)	Flow rate (mL/min)[a]	Change in mass sensitivity
4.6	1.66×10^{-1}	1000	1.0
2.0	3.14×10^{-2}	189.0	5.3
1.0	7.85×10^{-3}	47.3	21.1
0.05	1.96×10^{-5}	0.12	8469

a Flow rate (mL/min) = Linear Velocity (cm/min) x Cross-sectional area (cm^2). Linear Velocity for all columns is 6.02 cm/min.
b Assumes the same mass is injected while using the same linear flow velocity. The change in mass sensitivity is defined as the ratio of cross-sectional area compared with the conventional 4.6 mm i.d. column. Source: Bradshaw 1998.

Microelectrode detection

With the development of micro-separations, detectors were miniaturized to accommodate the extremely low volumes. Electrochemical detection (ECD) is successful in conventional scale separations due to its ease of implementation, selectivity and high sensitivity. ECD is a concentration sensitive technique; however, mass-detection limits improve as the volume of the microcolumn decreases. Therefore coupled together LC-EC is a valuable technique in analysis of volume limited samples. Of the three basic modes of ECD (amperometry, conductimetry and potentiometry), amperometry is the most commonly used due to its ease of implementation. In the amperometric mode, a constant potential is applied at the electrode and compounds are oxidized or reduced due to loss or gain of electrons. The current due to the flow of electrons is recorded and is a direct measure of the concentration of analyte present. For

a micro-electrode at steady state conditions, the equation for the limiting current is described by the equation:

$$i_{lim} = 4\ rnFDC$$

where r is the radius of the electrode, n is the number of electrons per mole transferred in the electrode reaction, F is Faraday's constant (96485 C/equivalent), D is the diffusion coefficient and C the concentration of the analyte. Conversion efficiencies are best when the electrode is placed inside the capillary because convection currents and analyte diffusion outside the capillary is avoided, Figure 1-7. Conversion efficiencies can be determined by application of Faraday's Law

$$Q = nFN$$

where Q is the total charge passed, determined from the peak area, n is the number of electrons per mole transferred in the process and N is the amount of analyte oxidized or reduced at the electrode. The amount of analyte injected can be calculated from the injection time, dead time, and column volume and analyte concentration (Knecht et al., 1984).

Research Overview

The research presented here is focused on bioanalytical method development using capillary liquid chromatography (50 μm i.d. capillaries), and electrochemical detection of OPA derivatives following preconcentration and gradient elution. The work aims to develop an off-line method to monitor different classes of amine-containing neurotransmitters (amino acids- chiral and non-chiral, catechol and indole amines) following microdialysis sampling of the striatum. However, the developed method is shown to be suitable for dealing with the complex nature of samples from different

biological sources. The method makes it possible to determine a wide variety of compounds in one analysis, gives quantitative and qualitative information in the study of neurotransmitter dynamics and allows for the possibility of discovering unexpected compounds.

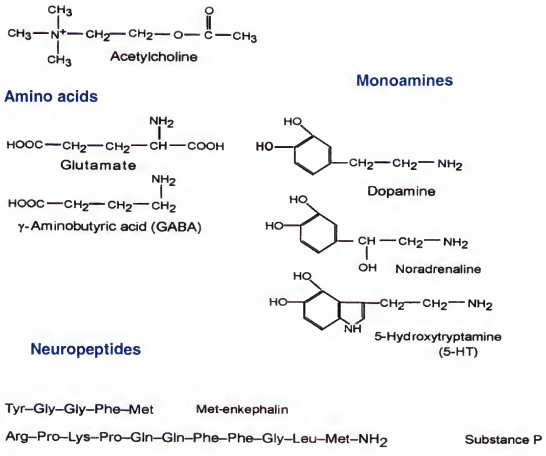
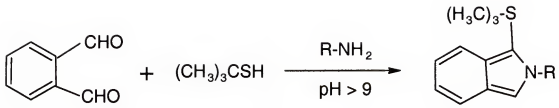


Figure 1-1 Structures of the most studied small molecule neurotransmitters and neuropeptides in the CNS



OPA **t-BuSH** **1-(alkylthio)-2-alkylisoindole-**

Figure 1-2 Reaction of OPA at high pH with a primary amine in the presence of tert-Butyl thiol (t-BuSH)

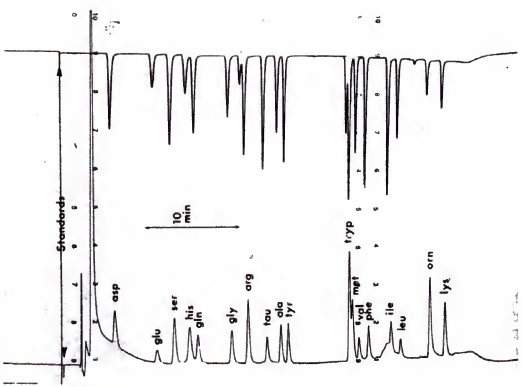


Figure 1-3 Amino acids derivatives of OPA and mercaptoethanol illustrating fluorescent (top trace) and electroactive properties (bottom) at 0.5 V. Source: Joseph and Davies (1983).

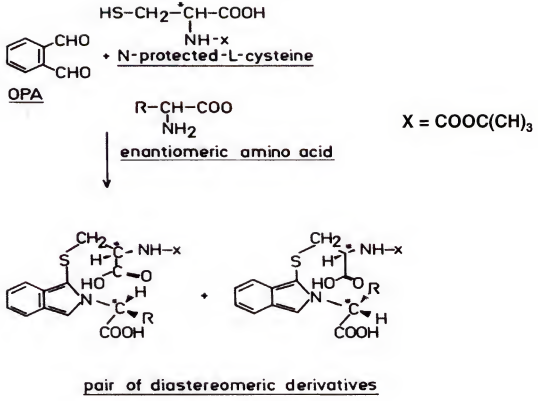


Figure 1-4 Reaction of OPA and a chiral amino acid in the presence of a chiral thiol (Boc-L-Cys) to form a pair of diastereometric derivatives. Source: Linder (1988).

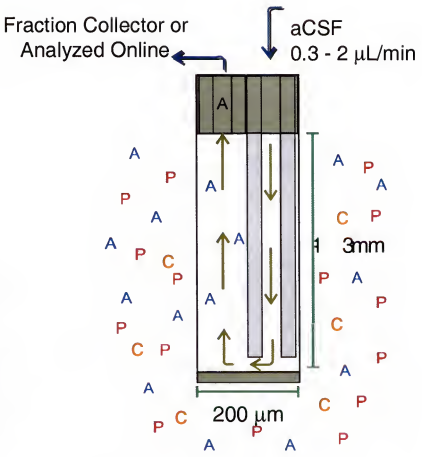


Figure 1-5 Schematic of a side-by-side microdialysis probe. The letters A, C, and P represents different molecules in the extracellular space. The probe is selective to molecules below the molecular weight cut off of the membrane surrounding the probe.

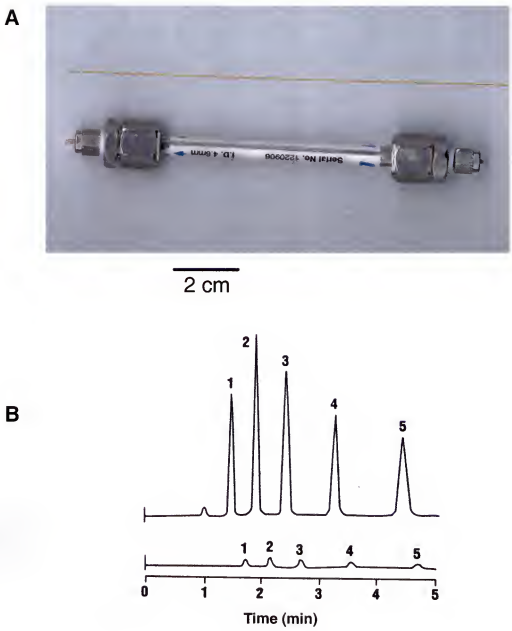


Figure 1-6 Illustration of Mass Sensitivity (A) Picture of a capillary column used in this work and a conventional 4.6 mm i.d column. (B) Mass sensitivity enhancement (factor of 20, Table 1-3) when the same sample volume was injected onto a 1 mm i.d and a 4.6 mm i.d column.

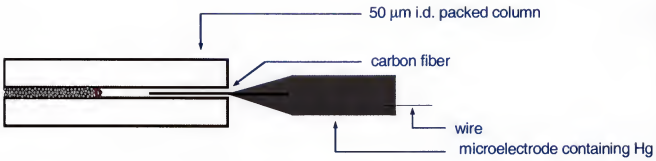


Figure 1-7 Carbon fiber microelectrode (9 µm o.d) inserted using a micropositioner into the outlet of a capillary column (50 µm i.d). An electrical connection is made between the carbon fiber and silver wire (connected to the current amplifier) using mercury.

CHAPTER 2
AUTOMATED CAPILLARY LC-EC FOR SIMULTANEOUS DETERMINATION OF NEUROACTIVE AMINES AND AMINO ACIDS

Introduction

Biogenic amines such as catecholamines (noradrenaline (NA) and dopamine (DA)) or indoleamines (5-hydroxytryptamine (5-HT)) and amino acids (aspartate (Asp), glutamate (Glu), γ-aminobutyric acid (GABA), and glycine (Gly)) are important classes of neurotransmitter (Cooper et al., 1986). In addition, other amino acids such as taurine (Tau), arginine (Arg), and citrulline (Cit) act as neuromodulators or indicators of neuronal activity. Because of the importance of these compounds, they are frequently measured in tissue extracts or in fractions collected from *in vivo* microdialysis sampling probes for neuroscience applications.

Neurotransmitter amino acid analysis is often performed by liquid chromatography with electrochemical (LC-EC) or laser-induced fluorescence detection (LIFD) following derivatization of the amino group with o-phthalaldehyde (OPA) and a thiol (Cheng et al., 1992; Keating et al., 1993; Cooper et al., 1994; Jensen and Marley, 1995). In 1971, Roth demonstrated that primary amines react with o-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol (β-ME) in an aqueous alkaline medium to yield fluorescent 1-alkylthio-2-alkyl-substituted isoindoles (Roth, 1971). OPA/β-ME derivatives of amino acids are readily oxidized at moderate potentials, permitting the use of electrochemical detectors (Joseph and Davies, 1983). However, the variable stability of OPA/β-ME derivatives makes the method insufficient for adequate analytical precision

(Chen et al., 1979; Buteau et al., 1981; Lee and Drescher, 1978). The stability of many OPA-derived isoindoles is now known to be influenced by a number of experimental parameters including thiol structure, thiol concentration, amine structure, solvent composition and pH (Jacobs et al.,1986; Allison et al., 1984; Stobaugh et al., 1983). Upon substitution of bulky thiols, fluorescence efficiency dropped significantly while redox behavior remained relatively insensitive to change in thiol structure (Allison et al., 1984). The use of t-BuSH as the thiol results in a stable isoindole derivative, yields an approximate two-fold improvement in electrochemical response, and allows for sensitive determination of amino acids in complex matrices without concerns regarding degradative loss (Allison et al., 1984, Simmons and Johnson, 1977; Smolders et al., 1995; Qu et al., 1998). For example, the half- life of GABA derivatized with OPA/β-ME is 8 minutes compared to 410 minutes using OPA/ t-BuSH (Allison et al., 1984; Jacobs, 1986). The use of t-BuSH as thiol in the OPA reaction yields stable derivatives for many amino acids with a good electrochemical response, making it a popular reagent for LC-EC based methods of neurotransmitter amino acid analysis. However, it has not been used with fluorescence detection because it results in derivatives with low fluorescence yield compared to other thiols.

In addition to OPA, 2,3-naphthalenedicarboxaldehyde (NDA) has proven to be a useful reagent for detection of amino acids by either fluorescence or electrochemistry (de Montigny et al., 1987; Roach and Harmony, 1987; Oates et al., 1990). More recently, the fluorogenic reagent 5-furoylquinloine-3-carboxaldehyde (FQ) has been utilized with capillary electrophoresis and laser-induced fluorescence detection (CE-LIF) for simultaneous analysis of amines and amino acids in dialysate samples (Chen et al., 2001).

Catecholamines and indoleamines are naturally electroactive and usually are measured by amperometry following separation by LC or CE (Cooper et al., 1994; Qian et al., 1999; Wallingford and Ewing, 1989; Newton and Justice, 1994). Determination of catecholamines by CE-LIF detection following derivatization of the amine functionality with NDA has also been reported (Robert el al., 1995; Gilman and Ewing, 1995).

It is often of interest to detect both biogenic amines and amino acids in biological samples for studies of interactions of neurotransmitters or drug effects on multiple targets; therefore, it would be useful to develop a method for simultaneous detection of neuroactive amines and amino acids. While CE-LIF with NDA derivatization has been used to detect both amino acids and catecholamines, the conditions used are different necessitating two separate analyses to detect both classes of compounds (Robert et al., 1995; Gilman and Ewing, 1995; Gamache et al., 1993; Bert et al., 1996; Soblosky et al., 1998). FQ has been utilized with CE-LIF for simultaneous determination of dopamine and amino acids (Chen et al., 2001), however, the detection limit obtained for dopamine was not sufficient to measure this compound at basal levels in dialysate samples.

In a previous report, the use of OPA/t-BuSH derivatization coupled with capillary LC-EC was demonstrated for amino acid neurotransmitter analysis (Boyd et al., 2000). The use of t-BuSH in this reaction is especially useful as it results in hydrophobic derivatives which can be preconcentrated on the column. With on-column preconcentration, as well as care in eliminating background peaks, detection limits less than 1 nM following injection of 250 nL samples has been achieved for all of the neuroactive amino acids (Boyd et al., 2000). In this work, we have investigated the use of capillary LC-EC with OPA/t-BuSH derivatization for simultaneous determination of

catecholamines, indole amines and amino acids in neurological samples. These neurotransmitters represent different classes, the separation of which has not been reported in the same run using the same solvent system. It was found that only small modifications were necessary to extend the method of Boyd to include catecholamines and indoleamines. We will demonstrate that the same principle of derivatization, separation by LC and EC detection can be used to separate different classes of neurotransmitter amines in a single experiment.

Experimental

Reagents and Buffers

Boric acid, OPA, t-BuSH, amino acid and biogenic monoamine standards were obtained from Sigma (St. Louis, MO). Iodoacetamide (IAA) and tetrabutylammonium perchlorate (TBAP) was from Fluka (St. Louis, MO). Phosphate buffer salts, sodium hydroxide, ammonium acetate and ethylenediamine-tetraacetic acid (EDTA) were enzyme or ACS grade and were obtained from Fisher Scientific (Atlanta, GA). Water, acetonitrile and methanol were HPLC grade and obtained from Burdick and Jackson (Muskegon, MI).

Stock solutions (10 mM stored at –20°C) of neurotransmitter standards were prepared in 100 mM HCl containing 0.5 M ascorbic acid and diluted prior to use. Dilutions of the standards to 10, 50, 100, 400 nM or other concentration were prepared daily using HPLC grade water. Excess ascorbic acid was added to the stock solution to prevent auto-oxidation of NA, DA and 5-HT before derivatization. This allowed the same stock solution of neurotransmitters, when stored at -20°C to be used up to 5 days. 50 mM phosphate buffered mobile phase at pH 6.5 was prepared by dissolving 4.22 g

Na_2HPO_4 and 4.73 g NaH_2PO_4 in 1 L water. 0.6 M boric acid solution adjusted to pH 10.5 was used as the derivatization buffer. Buffers were prepared using HPLC grade water and filtered using 0.22 μm carbon activated Teflon membranes (Alltech, Deerfiled, IL) and glass vacuum filtration system.

Derivatization Procedure

40 mM OPA/50 mM t-BuSH reagent was made by dissolving 26.8 mg OPA in 2.5 mL filtered methanol and adding 28.1 μL t-BuSH and 2.5 mL borate buffer (0.6 M). The solutions were stored at room temperature in darkened borosilicate glass vials that had been cleaned using 1 M HCl followed by rinses with HPLC grade water and absolute ethanol (Boyd et al., 2000). Microscale derivatization was performed using a Famos autosampler (LC Packings, San Francisco, CA) to deliver reagents. Unless stated otherwise, derivatization was as follows, 0.6 μL OPA/t-BuSH was added to 2 μL samples, mixed and allowed to react for 5 minutes. When derivatization is carried out, a large excess of reagents is needed to obtain fast reaction kinetics, For each sample derivatized, the concentration of OPA and t-BuSH (OPA: thiol /1:1.25) remained constant and allowed for at least a 10-fold excess over the total amino acid concentration (consider a mixture of 20 amino acids, each at a concentration of 8×10^{-6} M corresponds to a total concentration of 1.6×10^{-4}M). OPA is capable of reacting directly with primary amines in the absence of thiol to produce non-electroactive products (Jensen and Marley, 1995). For this reason, the OPA and thiol are mixed before addition of the sample containing primary amines. The excess reagent along with impurities in the reagent or solvent, and the formation of side products may result in interfering peaks in the chromatogram. Excess thiol was removed by adding 0.4 μL of 1 M IAA (935 mg in 5

mL methanol) and allowed to react for 3 minutes. All samples were derivatized in 250 µL tapered polypropylene microvials that had been pre-cleaned (Boyd et al., 2000).

Capillary Liquid Chromatography

The capillary LC system, illustrated in Figure 2-1 is similar to that previously described (Boyd et al., 2000; Oates and Jorgenson, 1989; Boyd and Kennedy, 1998). Capillary LC columns consisted of 50 µm i.d. x 30-34 cm long fused silica capillaries (Polymicro Technologies, Phoenix, AZ) slurry-packed with 5 µm Alltima C8 particles (Alltech, Deerfield, IL) by a previously described technique (Kennedy and Jorgenson, 1989). Mobile phase was delivered at 40 µL/min using two high-pressure syringe pumps (100 DM, ISCO, Lincoln, NE) with approximately 90% of the flow being carried to waste by a splitter (25 µm i.d. x 60 cm long) thus generating a backpressure of approximately 3500 psi. Injections were performed by the autosampler (Famos), which contains a 6-port injection valve (Valco C2) fitted with a 1 µL injection loop. To reduce the dead volume of the system, the capillary column was threaded through the injection valve port to the rotor. Mobile phase A was 50 mM phosphate buffer pH 6.5 containing 1 mM EDTA while mobile phase B was 35% phosphate buffer and 65% acetonitrile. Mobile phase solutions were degassed prior to loading the syringe pumps by sparging with He for at least 10 minutes.

Electrochemical Detection

The working electrode was a carbon fiber microelectrode (9 µm diameter by 1 mm length) fabricated using previously described methods (Kawagoe et al., 1993). The electrode was inserted using a micropositioner into the outlet of the capillary column mounted in an electrochemical cell containing 0.1 M KCl as supporting electrolyte.

Working electrodes were poised at + 0.75 V versus Ag/AgCl reference electrodes and were pretreated to improve reproducibility and reduce background current by sweeping the potential from 0 to 1.8 V at 1 V/s for 30 seconds (St. Claire and Jorgenson, 1985). Current was amplified using a Stanford SR-570 current amplifier (Sunnyvale, CA) set at 1 Hz low pass filter. The signal was digitized using a 16-bit AT-MIO data acquisition board (National Instruments, Austin TX) in a 486 DX computer with 5 Hz collection rate.

Cyclic voltammograms (CVs) were collected with an EI-400 potentiostat (Ensman Instrumentation, Bloomington, IN) interfaced to an IBM-compatible personal computer via a multifunction board. The current was measured in a two-electrode system with a Ag/AgCl reference electrode and a carbon fiber disk microelectrode (9 μm diameter). CVs were recorded from –500 to 1100 mV at a scan rate of 40 V/s in a partial aqueous background electrolyte solution of 50 % CH_3CN / 50 % 50 mM TBAP, 0.10 M acetate buffer, pH 5.75 by flow injection analysis (FIA). Background subtraction was used to remove the large charging currents associated with the use of high scan rates (Bath et al., 2002). Steady state voltammograms were recorded at 0.4 V/s in a background electrolyte of 100 mM TBAP in CH_3CN.

Microdialysis

Male Sprague-Dawley rats (250 –350 g) were anesthetized with a 1.0 mL subcutaneous injection of 0.1-g/mL chloral hydrate (Sigma) and mounted in a stereotaxic frame before surgery. Microdialysis sampling was performed using side-by-side 3 mm long probes constructed in-house using methods described elsewhere (Pettit and Justice, 1991). Probes were inserted at a rate of 500 μm/min to minimize tissue damage and were implanted at + 0.02 AP, - 0.30 ML and - 0.65 DL from bregma to sample the striatum

(Paximos and Watson, 1997). Artificial cerebral spinal fluid (aCSF) (145 mM NaCl, 2.68 mM KCl, 1.01 mM $MgSO_4$ and 1.22 mM $CaCl_2$) was perfused through the probe at 0.3 μL/min using a micro-syringe pump (CMA/102, Acton MA). Sample collection began after basal levels were constant, about 2 hr after probe implantation. Fractions were collected at 7-minute intervals (2.1 μL per fraction) and stored at –50 °C. To quantify the concentration of the dialysate, a standard curve consisting of 10, 50, 100, 400 and 1000 nM neurotransmitters was constructed. Standards and samples were treated identically and analyzed on the same day. In vitro microdialysis probe recovery was determined before an *in vivo* experiment at 0.3 μL/min.

Results and Discussion

Detection and Derivatization Conditions

Previously, the separation and high-sensitivity detection of 16 amino acid neurotransmitters using 50 μm i.d. capillary columns packed with C-8 5 μm particles has been demonstrated (Boyd et al., 2000). A two-step derivatization process was used. In the first step, the OPA/t-BuSH (1:1.25 molar ratio) reagent was used to form electroactive 1-alkylthio-2-alkylisoindoles. IAA was immediately added after the first reaction as this step has previously been found to reduce background peaks (Allison et al., 1984; Boyd and Kennedy, 1998). Amino acid derivatives were separated at 2500 psi using an initial mobile phase of 65% A and 35% B with a linear gradient of 2% B/min over 5 minutes, then 1% B over 10 minutes, Figure 2-2. After elution of the amino acids, the mobile phase was stepped to 100% B. In preliminary experiments using the above conditions, we found that OPA/t-BuSH derivatives of NA, DA, histamine and 5-HT eluted well after the most hydrophobic amino acids when the mobile phase was stepped

to 100% mobile phase B. A chromatogram illustrating detection of the amines with the amino acids under these conditions is illustrated in Figure 2-3. Present in the blank are additional background peaks which were found to co-elute with histamine and dopamine. Further experiments did not include histamine as this peak was often obscured and not positively identified in all cases (peak proved difficult to reliably resolve from background peak) and labeling omitted from some of the remaining Figures.

Isoindole derivatives formed from catecholamines and indoleamines are highly hydrophobic resulting in very long separation times and requirement of a very high organic mobile phase for elution. In addition, the slight tailing of NA and DA might be as a result of strongly partitioning into the bonded stationary phase. Attempts at better quantitation and resolution of DA and histamine, include use of a series of other stationary phase materials in making capillary columns. Investigated using the same mobile phase and gradient conditions were OPA-HR particles (gives significant tailing of all analytes except NA, DA and 5-HT); YMC Basic (consists of mixed mode, its bonding chemistry is based on a combination of C-2, C-4 and C-8 alkyl chains), results in co-elution of Cit and Arg. Another type, based on shorter alkyl chains, Alltima C-4 5μm was used. With this material, Asp and Glu, being negatively charged did not partition well into the stationary phase, did not preconcentrate well and were observed as one broad peak in the chromatogram, Figure 2-4. Various buffer combinations were investigated, however, phosphate buffer was found to give the best separation of OPA derivatives compared to borate or citrate; phosphate buffer (50 mM) gives optimal separation, however when used with acetonitrile above 65 % precipitation occurs (Lindroth and Mopper, 1979).

The S/N obtained for NA, DA and 5-HT (250 nM, 250 nL injected) was investigated using hydrodynamic voltammetry (HDV) in the potential range of 0.50 - 0.95 V. The S/N was increased with an increase in potential (0.50 - 0.65V) then level between 0.70 – 0.85V. At potentials, above 0.95 V, the S/N was greatly reduced and additional peaks appeared in the chromatogram. It was found that the detection potential that gave the best signal to noise (S/N) for NA, DA and 5-HT was +0.75 V, which was the same as that found previously for amino acids (Boyd and Kennedy, 1998; Boyd et al., 2000). The time required for OPA/t-BuSH to react with NA, DA and 5-HT (500 nM of each) was determined by varying the reaction time (prior to addition of IAA) from 1-10 minutes. Above 2.5 minutes the peak areas were independent of reaction time, indicating complete reaction within this time. For testing the stability of the DA derivative (500 nM standard), derivatization was done by reacting OPA/t-BuSH with 2 μL DA, and scavenging with IAA. An aliquot of 1 μL was withdrawn and chromatographed (injected, separated and the column re-equilibrated, total time = 40 minutes). Immediately afterward, 1 μL of the same derivatized solution was withdrawn and analyzed (derivatized solution sat at room temperature for 40 min). Peak areas obtained from the two chromatograms were within 5% of each other (n=5) indicating good stability of the derivatives. Similarly, after the reaction of OPA/t-BuSH with DA (500 nM standard), the absorbance maxima monitored by UV-Vis at 296 nm over 60 minutes remained unchanged. Thus, the DA derivative produced is stable for at least 40 -60 minutes.

Problems with Initial Conditions

While good signals were obtained for NA, DA, and 5-HT at 100 nM, Figure 2-5A, it was impossible to identify peaks corresponding to these compounds at 10 nM due to the highly irregular baseline, Figure 2-5B. In contrast, the signal-to-noise ratio (S/N) for the amino acids even at 5 nM was still high (Figure 2-5C) primarily due to a more regular baseline as seen by comparing the insets of the Figure. The separation time could be decreased without loss of resolution when the gradient was optimized using Linear Solvent Strength (LSS) Theory (Snyder et al., 1970). The result was a minor modification of the gradient elution method to 4% B/min over 8.5 minutes followed by a step change to 100% B employed for all other experiments.

It was hypothesized that increased noise in the baseline during elution of the amines (NA, DA, 5-HT) was due in part to elution of hydrophobic compounds present in the sample or a mobile phase component. An increase in the baseline could occur as a result of a decrease in conductivity by the use of high organic or the sudden change encountered by the electrode as a result of a step gradient as opposed to a linear gradient. To ameliorate this problem, the mobile phase and derivatization solutions were cleaned using a filter containing activated carbon - a Super Clean Mobile Phase Purification Filter (Alltech, Deerfiled IL), solvent artifact peaks present in the blank were reduced. In addition, the mobile phase gradient was changed to 4% B/min for the first 10 min followed by 10 % B/ min until 100 % mobile phase B was achieved thus eliminating the use of a step change prior to elution of NA, DA, and 5-HT. The use of a linear gradient lowered noise by reducing the detrimental effects of a sudden change in mobile phase composition as well as improving resolution of background components. With these modifications, the peak-to-peak noise was reduced ~4-fold (from 8 to 2 pA, see insets of

Figure 2-5. While an improvement, these noise levels were still over 3 times the levels during the elution window of the amino acids.

A third change that improved the S/N was increasing the borate concentration in the derivatization media from 33 to 70 mM which improved the signal by ~2-fold for the biogenic amines (an increase in peak height by 1.8, 1.5 and 1.3 for NA, DA and 5-HT respectively, a slight decrease for Asp and Glu, the other amino acids were virtually unaffected by the increase in borate). The borate concentration in the derivatization and separation medium was shown to be of utmost importance for the separation of catecholamines (Bert et al., 1996). Further increases above 70 mM resulted in no improvement. A similar effect has been reported previously with NDA derivatization of NA and DA and may reflect improved derivatization yields (Denroy et al., 1998). These combined modifications improved the detection limit ~8-fold for the catecholamines and indoleamines. With these improved conditions, the 10 nM standards were readily detected, Figure 2-5 D.

Reproducibility, Linearity and Detection Limits

Figure 2-6A, which depicts a chromatogram resulting from injection of a mixture containing the target neuroactive compounds Asp, Glu, Arg, Cit, Gly, Tau, GABA, NA, DA, and 5-HT demonstrates that these compounds were well resolved from each other and background peaks using the new separation conditions. Histamine could usually be detected under these conditions; however, its proximity to a large background peak sometimes prevented its quantification. Regression analysis using either peak height or area versus concentration demonstrated linearity from 10 nM to 10 μM, with r^2-values

0.99 or better for all primary amines. The precision of the method, evaluated using a 50 nM standard following triplicate runs is summarized in Table 2-1.

Detection limits of 0.3 – 0.9 nM (80 - 200 amol) were obtained for amino acids while detection limits for NA, DA and 5-HT were 1 – 4 nM (200 - 900 amol). The detection limit was calculated as the concentration required to give a signal-to-noise ratio of 2, where the noise is measured as peak-to-peak (p-p) noise in the chromatogram in the region where the amines elute. These detection limits reflect the lowest concentration that can be derivatized and detected (dilution factor upon derivatization was not included in the calculation, sample was diluted 2/3 upon dilution). Using our technique, uniformly low LODs are obtained indicating no discrimination took place during reaction to form electroactive derivatives. Detection limits for NA, DA and 5-HT listed in Table 2-1are a bit worst than those previously reported using CZE with LIF or electrochemical detection; 10 amol reported for NDA labeled derivative of NA (Gilman and Ewing, 1995), 1.8 amol reported for of NA and DA (Bert et al., 1995). Catechol and indole amines are naturally electroactive and can be detected without a derivatization procedure, detection limits realized are reported to be 3.0, 2.4 and 0.7 amol for NA, DA and 5-HT respectively (Wallingford and Ewing, 1989). Although other techniques may offer better limits of detection for one or more of these compounds, this is the first technique that enables determination of both classes of neurotransmitters at low nanomolar levels without the need for sample splitting or column switching.

The sensitivity for amino acids and catechol amines were similar under the conditions used. Thus, injection of 30 fmol of Gly, GABA and DA resulted in peak areas of 1.2, 1.1 and 1.2 nC respectively. (Conversion efficiencies were determined using

Faraday's Law, $Q = nFN_{det}$, where Q is charge in Coulombs determined from the peak area, n is the number of electrons per mole transferred in the process, F is Faraday's constant and N_{det}, the number of moles detected. The number of moles injected, N_{inj} was calculated from the injection volume and the concentration). Peak areas corresponds to conversion efficiencies (given in Table 2-2) of about 40 % for the amino acids assuming a 1-electron transfer reaction for each OPA/t-BuSH derivative. Injection of 30 fmol of DA also resulted in a peak area of 1.2 nC. Thus, the signal obtained for derivatized DA is about 1/3 that expected based on the conversion efficiency seen for the amino acids with this system. That is, if a 3 e^- transfer reaction is assumed (1 electron for the isoindole functionality and 2 electrons for the catechol functional group), this corresponds to a conversion efficiency of just 14%. Indeed, underivatized DA yields a better signal (3.3 nC for 30 fmol injection) than derivatized DA. Underivatized DA was detected at +0.75 V *vs* Ag/AgCl after isocratic elution with 7 % CH_3CN in 50 mM phosphate buffer, 4 mM heptane sulfonate, 1 mM EDTA, at pH 3.7 using a 50 μm i.d. x 15 cm long column packed with Alltima C18 μm particles and 3450 psi.

Several hypotheses were tested to explain the lower than expected signals for DA including: 1) formation of multiple products during derivatization, 2) incomplete reaction of DA under the conditions used, and 3) chemical conversion of DA during derivatization to a product that does not generate a 3-electron transfer reaction. As only a single peak for derivatized DA was detected, no evidence supporting the first hypothesis was found. The second hypothesis was eliminated by the observation that no underivatized DA could be detected after the derivatization reaction indicating that all of the DA was consumed

under the conditions used. Evidence supporting the third hypothesis was obtained by cyclic voltammetry (CV) of DA before and after derivatization.

CV of underivatized DA at 40 V/s gave the expected result with a peak at 700 mV on the anodic scan, corresponding to oxidation of DA, and a peak at –100 mV on the cathodic scan corresponding to reduction of quinone formed by dopamine oxidation, Figure 2-7A. CV of derivatized dopamine revealed a different profile, with two peaks on the anodic scan, one at –200 mV (likely due to pre-adsorbed species), the other at 800 mV (oxidation of the OPA product), Figure 2-7B. No waves were observed on the reverse scan indicating an irreversible oxidation. The complete lack of a reverse (cathodic) wave for derivatized DA suggests that the product of the OPA/t-BuSH reaction with DA does not contain a catechol functional group. Irreversible voltammetry is characteristic of the isoindole moiety and is similar to that observed for OPA derivatives of amino acids (Allison et al 1984). Under the same condition, the CV of Gly (underivatized and derivatized) were recorded, Figure 2-7(C,D). If we assume that the derivatization product is similar for DA and Gly, then we would expect a similar wave pattern, this is in fact our observation (comparing Figures 2-7B and 2-7D). Thus, the decrease in sensitivity observed for catecholamines upon derivatization is due to loss of the catechol functional group during derivatization resulting in amperometric detection of only the isoindole group. Figure 2-8 shows typical steady state CVs recorded at 0.4 V/s for derivatized DA, ferrocenecarboxylic acid (Fecp-COOH), a one electron oxidant used as a standard and underivatized DA each at 500 μM. Measurement of the limiting current revealed that underivatized DA yielded a 2-electron oxidation, which is expected for a catechol. In contrast, the limiting current of OPA derivatized DA yielded a 1-

electron oxidation (this calculation assumes that all of the DA was derivatized) when compared to Fecp-COOH. The voltammetric data indicate that the product formed from the OPA reaction does not give the simple electrochemical response expected for the catechol and 1-alkylthio-2-alkyl-substituted isoindole (3-electron transfer). Rather, the electrochemical response resembles that for the amino acids derivatives. The reaction of OPA with primary amines requires high pH, in particular, we found that CVs of underivatized DA recorded in a background solution of 400 mM borate buffer pH 10.5 did not reveal waves for the catechol group (data not shown). Such a result suggests that the reaction chemistry involves degradation or alteration of the catechol functionality during the reaction at high pH. The CV data suggest that the derivatization product is an isoindole with no catechol group resulting in amperometric detection of only the isoindole group. The peak area for comparable injected quantities is therefore expected to be the same for amino acids and the other primary amines, in agreement with experimental data of this work. Further chemical characterization would be necessary to identify the product of the DA reaction with OPA; perhaps involving identification of the products by mass spectrometry would help to elucidate the cause of the weaker signals.

Applications

Microdialysis was previously used in this lab to quantitate extracellular amino acid neurotransmitters and to correlate changes in their concentration with pharmacological manipulations (Boyd et al., 2000). Temporal resolution was of utmost importance, probes were perfused at 1.2 μL/minute, fractions collected at 10-s intervals and diluted to 2μL prior to derivatization. However NA, DA and 5-HT could not be detected because of their low nanomolar extracellular concentrations compared to amino

acids, which typically have levels of > 100nM in diluted dialysate and do not require the sensitivity needed to measure very low concentrations (Wages et al., 1986; Boyd et al., 2000). Slower perfusion rates typically gives higher recoveries, allowing for more concentrated samples to be removed from the extracellular fluid (Wages et al., 1986). The best conditions for simultaneous monitoring of amino acids plus catechol and indole amines appears to be conflicting, maximizing sample size while operating at low flow rates which gives smaller sample volumes per unit time.

To demonstrate the utility of the method, dialysate collected from the rat striatum was analyzed as an example of a typical neurochemical application. Figure 2-6 compares chromatograms for standards at 400 nM , a blank and a striatal microdialysis sample (collected at 7-min interval when the probe was perfused at 0.3 μL/min). The small flow rate and relatively long collection time was necessary to establish basal concentration levels of low nanomolar neuroactive amines. Peaks corresponding to Asp, Glu, Cit, Arg, Gly, Tau, GABA, Tau, NA, histamine, and DA were readily detected in the dialysate sample. As mentioned above, the proximity of the histamine peak to a reagent peak precluded quantification on all samples, however, it could be detected in many cases. Poor recovery of 5-HT and low concentration in the brain prevented its detection. While this method is capable of measuring the very low levels of NA and DA found in dialysate, some amino acids have levels over 1000-fold greater than NA and DA. As a result, amino acids such as Gly, Arg, and Tau are off-scale at the same gain settings needed to detect the lower level NA and DA. This problem can be readily solved by using a data acquisition system with a larger dynamic range or use of automatic gain control to adjust the gain as analytes elute. The basal dialysate concentrations obtained

for Asp, Glu, GABA, NA and DA are listed in Table 2-3. The basal concentrations for Asp, Glu, GABA, and DA are consistent with those reported previously at similar flow rates (Boyd and Kennedy, 1998; Moriari et al., 1993; Semba et al., 1995; Britton et al, 1996). NA has not been measured previously in the striatum at this flow rate; however, in a previous report in which NA and DA were both detected in dialysate samples of the rat striatum, the levels were found to be present at a nearly 1:1 ratio which is similar to that observed in this work (Dawson et al., 2000). Operating at a flow rate of 0.1μL/minute, the basal level of DA is estimated to be in the range 10-50 nM (Wages et al., 1986), we are therefore confident that our reported values for all amine neurotransmitters are within acceptable limits.

To demonstrate measurement during a pharmacological manipulation, the catecholamine uptake blocker nomifensine (30 μM) was added to the perfused a-CSF after basal levels were established. Following this treatment, considerable increases in DA and NA were observed, while Asp, Glu and GABA levels were decreased, Figure 2-9; however, the decrease in GABA was not statistically different from basal levels ($p > 0.05$). The increase in DA dialysate levels is well documented and is due to the increased recovery and overflow of DA (Zahniser et al., 2000; Davidson et al., 2002; Hauber and Fuchs, 2000; Peters and Michael, 2000). These results are in good agreement with previous work wherein perfused nomifensine was shown to considerably enhance DA and NA output in the striatum, 500 % and 240 % increase above basal for DA and NA respectively (Cenci et al., 1992). Very few studies have reported an *in vivo* interaction between dopamine and/or glutamate and/or GABA in the striatum. However, the characteristic of Glu-induced GABA release depends on experimental conditions such as

anesthetics, recovery time after surgery, ionic composition of perfusion medium and the brain region where the dialysate is collected (Drew et al., 1989). The effect on Glu and Asp may indicate a regulation of Glu/Asp release by DA. Our results appear to be in good agreement with previous measurements. Using striatal neurons of rat brain, it was observed that events altering the neuronal activity of DA-containing neurons leading to increased release of DA should be associated with decreased responses (excitatory and inhibitory) relative to basal release (Chiodo and Berger, 1986). In another study, administration of drugs with DA-reuptake blocking properties (nomifensine or phencyclidine) did not alter the basal extracellular GABA levels in the striatum of freely moving rats (Honda et al., 1995). Phencyclidine, has properties similar to nomifensine, is shown to exert an inhibitory action on excitatory amino acid transmission in the striatum of freely moving rats (Lodge et al., 1983).

Conclusion

The method presented allows detection of amine and amino acid neurotransmitters with detection limits from 0.3 to 4 nM in 250 nL samples. Only minor modifications were required over the existing methods for amino acid analysis. The technique is ideal for analyzing small samples obtained from different biological sources. The reproducibility of derivatization, injection, migration and detection limits are satisfactory for biological or pharmacological studies requiring simultaneous monitoring of amine-containing neurotransmitters. The main limitation of the method is the lack of dynamic range to cover large differences in concentration typically found for amino acids and amines; however, this problem can be readily solved. In addition, the detection limit for 5-HT may not be sufficient to detect this compound in some dialysate samples. However, improvements in the recovery of 5-HT may improve this situation.

Nonetheless, these results illustrate the advantage of monitoring of multiple neurotransmitter classes by a single technique.

Table 2-1 Detection limits, reproducibility of retention time, peak area and peak height for neuroactive amines.

Amine NT	Ret time R.S.D (%).	Peak Height R.S.D (%).	Peak Area R.S.D (%).	[a]Detection Limit (nM)	Detection Limit (amol)
L-Asp	0.71	2.9	2.9	0.8	200
L-Glu	0.75	3.1	1.3	0.9	200
L-Cit	0.10	2.3	1.2	0.4	100
L-Arg	0.12	4.2	3.2	0.3	80
Gly	0.21	1.4	1.8	0.4	90
Tau	0.15	1.6	1.1	0.7	200
GABA	0.19	2.1	1.6	0.6	100
NA	0.33	4.6	3.9	1	200
DA	0.45	6.2	4.6	2	400
5-HT	0.53	6.3	4.3	4	900

[a] Calculated as the concentration corresponding to 2 x (S/N)/sensitivity. Evaluated using three consecutive analyses of a mix of 50 nM standards and injection volume of 250 nL.

Table 2-2 Conversion efficiencies calculated using Faraday's Law for several amine neurotransmitters.

Amine NT	N_{inj} (fmol)	peak area (pC)	n(assumed)	conversion efficiency (%)
Gly	30	1254	1	43
GABA	30	1116	1	39
DA	30	1202	1	41
DA	30	1202	3	14
DA[a]	30	3300	2	57

Conversion efficiencies calculated as the $(N_{det}/N_{inj})x100$

[a] Separations conditions differ for underivatized DA, detected at +0.75 V *vs* Ag/AgCl after isocratic elution with 7 % CH_3CN in 50 mM PO_4^- buffer pH 3.7, 1 mM EDTA using a 50 µm i.d. x 15 cm long column packed with Alltima C18 µm particles.

Table 2-3 Basal Concentrations (n = 8) and probe recovery (n = 5) of amine neurotransmitters in the rat striatum. Values are given as mean ± SEM.

Amine NT	Dialysate Basal Concentration (nM)	In Vitro Probe Recovery (%)
L-Asp	178 ± 34	51 ± 5
L-Glu	454 ± 66	61 ± 5
GABA	117 ± 16	58 ± 8
NA	31 ± 6	77 ± 3
DA	48 ± 5	81 ± 17
5-HT	ND	14 ± 1

ND – not detected

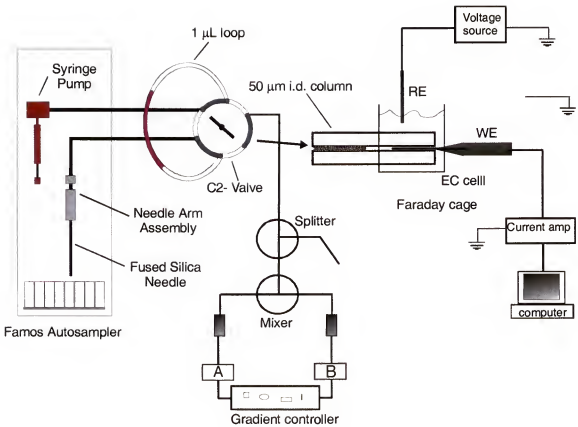


Figure 2-1 Schematic of the capillary LC-EC instrument. Small samples are derivatized using an autosampler. A plug of sample is sandwiched injected onto a 1 µL loop, then preconcentrated at the head of the capillary column. A gradient is applied; the analytes are separated and detected by amperometry at a carbon fiber microelectrode inserted inside the outlet of the capillary column. Conventional HPLC pumps are used which necessitates the use of a flow splitter to divert the majority of the flow to waste.

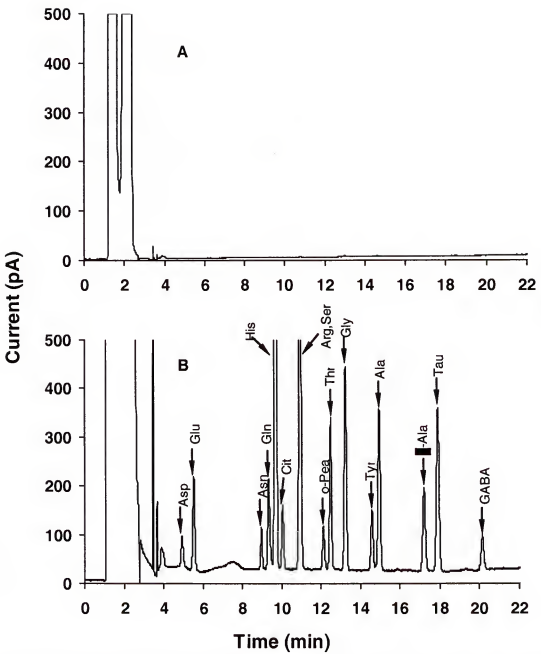


Figure 2-2 Chromatogram illustrating the separation of: (A) blank, (B)16 amino acids (200 – 500 nm) on a 27cm long x 50 μm i.d capillary column consisting of Alltima C-8 5μm particles. Flow rate through the column was 3 nL/sec, injection volume of 100 nL.

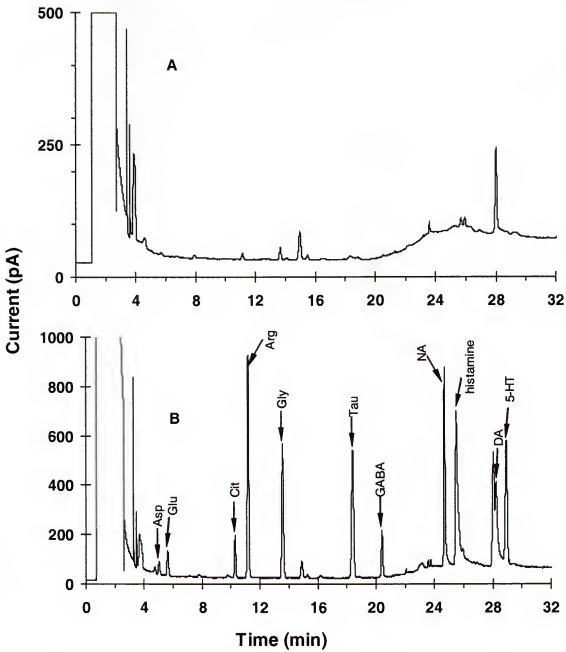


Figure 2-3 Chromatogram illustrating the separation of: (A) blank, (B) target amino acids and NA, histamine, DA and 5-HT. Derivatives were separated at 3 nL/s using the mobile phase gradient described in the text. Standards are at 100-300 nM and 100 nL were injected. Analytes were derivatized as described in text with 33 mM borate in the derivatization buffer.

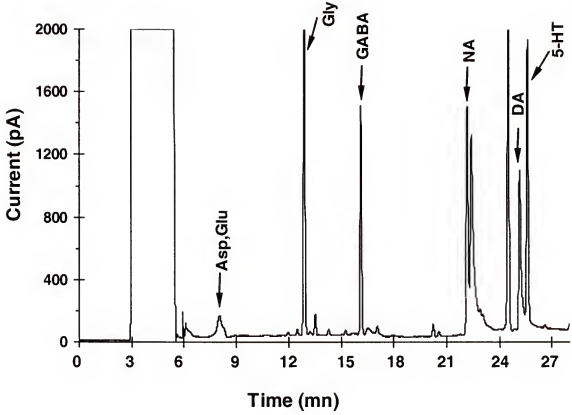


Figure 2-4 Chromatogram obtained for the separation of Asp, Glu, Gly, GABA, NA, HI, DA and 5-HT, each at a concentration of 500 nM on a 30 cm long column containing Alltima C-4 5 µm particles following injection of 100 nL. Separation was carried out at 2200 psi , at a flow rate of 3.2 nL/sec.

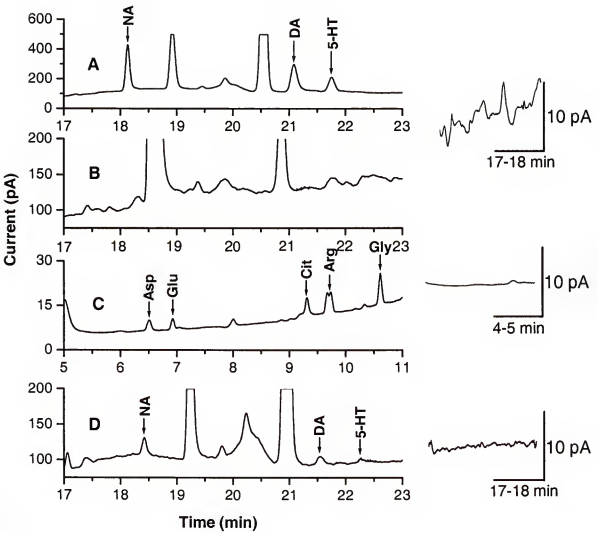


Figure 2-5 Chromatograms illustrating improvements in detection limit. (A) Chromatogram showing elution of NA, DA and 5-HT at 100 nM (250 nL injection) derivatized as outlined in the text. Derivatives were separated at 3.2 nL/s by a gradient elution initially at 35%B then increasing by 4% B/min over 7.5 minutes. (B) Same as (A) with analytes derivatized at 10 nM. Inset shows the irregular baseline that occurs in the elution region of the amines. (C) Detection of amino acids using the same conditions as (A) and (B) at 5 nM. Inset shows the lower noise of the baseline in this region of the chromatogram. (D) Chromatogram showing detection of derivatized NA, DA, and 5-HT at 10 nM using the modified method, which involved filtering the sample, shallower gradient, and addition of 70 mM borate to the derivatization media as, described in the text.

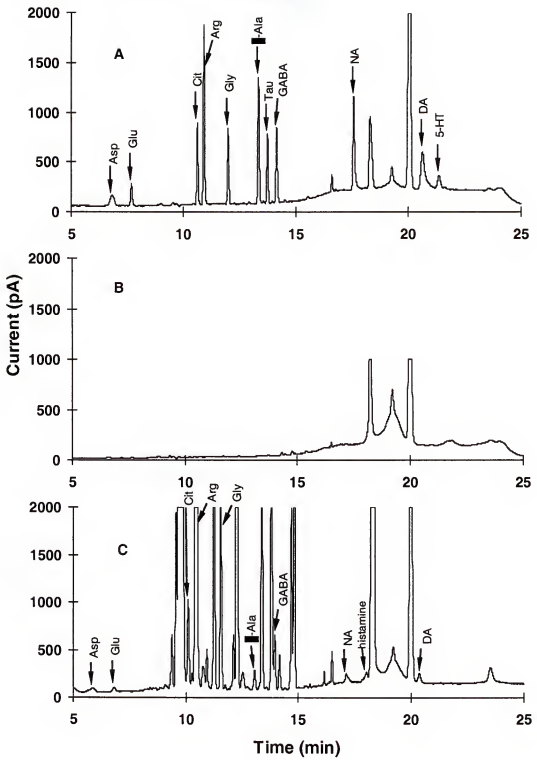


Figure 2-6 Chromatograms resulting from analysis of: (A) neurotransmitter standard, each at 400 nM, (B) blank, and (C) a microdialysis sample obtained from the striatum of an anesthetized rat.

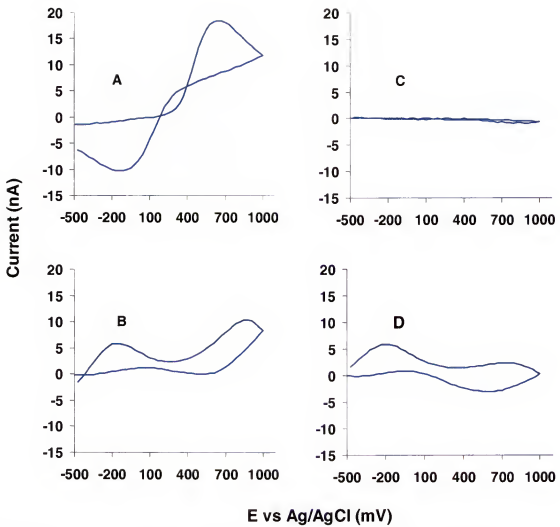


Figure 2-7 Cyclic voltammograms of (A) underivatized DA (B) derivatized DA (C) underivatized Gly (D) derivatized Gly each at a concentration of 500 μM in a background electrolyte solution of 50% CH_3CN/ 50% 50 mM TBAP, 0.1 M acetate at pH 5.75 at a scan rate of 40 V/s. Voltammograms were obtained in a flow injection apparatus and are background subtracted as described in the experimental section.

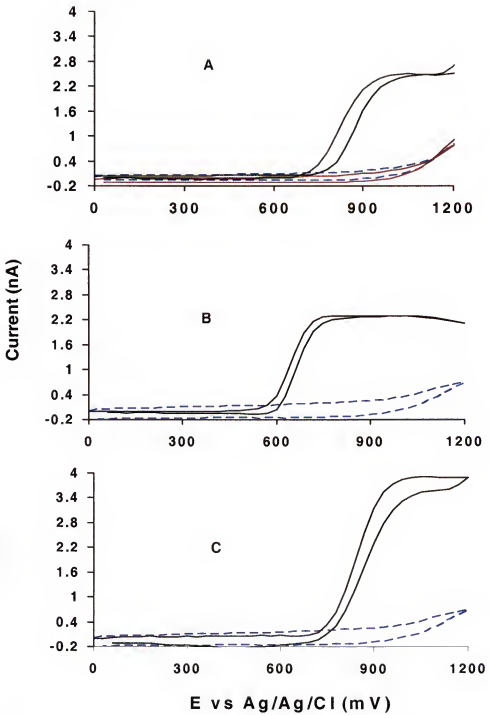


Figure 2-8 Steady state CVs recorded at 0.4 V/s for (A) derivatized DA (B) ferrocene carboxylic acid and (C) underivatized DA each at a concentration of 500 μM. Blue line represents the background electrolyte. In A the CV of OPA and thiol alone was recorded.

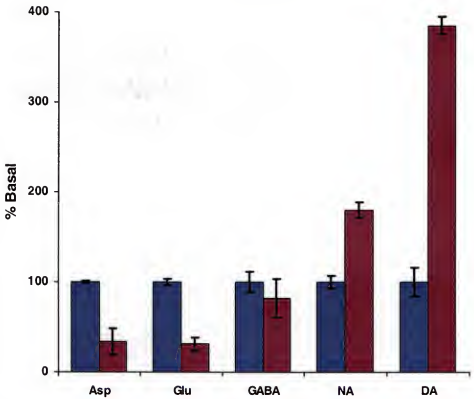


Figure 2-9 Comparison of selected neurotransmitter levels during *in vivo* microdialysis in the striatum before and after perfusion of the DA uptake inhibitor nomifensine (30 μM) for 45 minutes. Data are presented as the percent of the basal level (i.e., the level before addition of the drug) and represent the mean ± SEM for 4 rats.

CHAPTER 3
L-AMINO ACID ANALYSIS APPLIED TO NEUROTRANSMITTER RELEASE AND FUNCTION IN EMBRYONIC CORTICAL CELLS AND BUTTERFLY BRAINS

Introduction

During development, neurons are generated in specialized regions adjacent to the ventricle. Following terminal mitosis, neurons migrate from the ventricular zone, through the cell-sparse intermediate zone (iz) and into the cortical plate (cp) where they organize into layers and differentiate further (Jacobson, 1991). During embryonic development, γ-aminobutyric acid (GABA) is transiently expressed in many regions of the central nervous system (CNS) suggesting a functional role in development (Schaffner et al., 1993). *In vivo*, GABA is detected near the target destinations for migrating neurons. In addition, GABA has been found to promote movement of embryonic cells dissociated from the rat CNS in vitro (Behar et al., 1996). These results suggest that amino acids, such as GABA and Glu may guide newly generated neurons towards their final positions. This work was done in conjunction with a group at the Laboratory of Neurophysiology, National Institute of Health (NIH) to identify amine neurotransmitters secreted by the embryonic skeletal muscle during migration.

The knowledge of neurotransmitter receptors in insects is limited, however, over 60 neurotransmitter receptor sub-types have been documented including receptors for DA, 5-HT, GABA, and Glu (Kulagina et al., 2001; Roeder, 1994, Oshborne, 1996). There is however, strong evidence to suggest that in a number of species, neurotransmitters can regulate activity (Thompson et al., 1990; Kaatz et al., 1994). Brain

tissue samples from naïve (housed in cages) and wild butterflies were analyzed to possibly correlate the level and type of neurotransmitter present in each group.

Detection of amines in different biological samples is relevant to understand their roles in development or behavior. The off-line method developed in chapter 2 was applied to study amine-neurotransmitter release and function from different biological sources. Tissue samples (embryonic muscle cells and butterfly brains) were analyzed as examples of typical neurochemical applications.

Experimental

Muscle Cell Preparation

Cells from the cortical plate (cp) and ventricular zone (vz) of pregnant Sprague-Dawley rats at embryonic day 18 (E18) were used. Detailed preparation, dissociation (into single-cells suspension) and characterization can be found elsewhere (Behar et al., 2001). Cp and vz cells were suspended in 1 mL of a physiological saline solution (145 mM NaCl, 5mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES buffer, 10 mM glucose, pH 7.2) and incubated over time (30 mins, 2 hr, 4hr, 8hr) at 37 ºC. Cells were pelleted at 80 g for 10 min, the supernatants collected and stored at -20ºC until analyzed by capillary LC with gradient elution and electrochemical detection.

Butterfly Brain Tissue Preparation

Whole brains including optic lobes of Agraulis Vanillae L (Lepidoptera, Nymphalidae) were removed and homogenized in 0.5 mL water and centrifuged for 2 minutes at high speed. The supernatant was diluted with distilled water to a volume of 1.5 mL, filtered through sterile acrodisc 0.2 nm syringe filters (Fisher, Atlanta, GA) and stored at –50 °C prior to analysis by capillary LC with gradient elution and electrochemical detection.

Capillary LC-EC

Amine neurotransmitters (Asp, Glu, β-Ala, Tau, GABA) were determined by capillary LC with EC detection as previously described in chapter 2. Small aliquots (2μL) of supernatant were derivatized by addition of OPA/t-BuSH (0.4 μL). After reacting for 2 min, 1M IAA (0.4 μL) was added to scavenge the excess thiol. Immediately following derivatization, 250 nL of the sample was injected onto a capillary column, 50 μm i.d. x 36 cm long fused silica capillary packed with Alltima C8, 5 μm particles. In a second analysis, the same sample was diluted with HPLC water. Amine neurotransmitters were separated by gradient elution and detection by amperometry (0.75V). Mobile phase A was 50 mM phosphate buffer at pH6.5 and mobile phase B was a mixture of MPA (35 %) and acetonitrile (65 %). Gradient elution was initialized with 35% B for 1.5 min, and then linearly changed to 100 % B over 8.5 mins. Flow rate through the column was approximately 250 nL/min.

Results and Discussion

Muscle Cells

Cells from whole cortices of E-17 or E-18, vz and cp tissue segments were migrated to GABA or Tau using an in vitro chemotaxis assay- directed migration along a chemical gradient (Collaborative Research, Bethesda, MD). To identify cells expressing metabotropic $GABA_B$ receptors ($GABA_B$-R) coupled to Ca^{2+} or K^+ channels, embryonic cortical sections were immunolabelled with anti-$GABA_B$-R1 antibodies. Immunopositive cells were observed in the vz and cp regions, indicating that embryonic cells expressed $GABA_B$-R1 proteins. Cortices were microdissected into the immature cells of the ventricular zone (vz) and mature neurons of the cortical plate (cp) (Behar et al., 1998).

PT-PCR and Western blotting were used to examine the expression of $GABA_B$-Rs (for more details see Behar, 2001). Vz and cp tissue segments were dissociated into single-cell suspensions and immunolabelled with $GABA_B$.R antibodies before and after migration to GABA in vitro, Figure 3-1. The results indicate that migration to GABA results in an enrichment of $GABA_B$ receptors while only few cells, 10-20 % of the starting population were immunopositive for GABA receptors.

The Chemotaxis assay provided evidence that cortical cells release GABA or taurine that could influence neuroblast migration (Behar et al., 1994). The amino acids released spontaneously (no drug added to medium) from vz and cp cells suspended at the same density (one million cells incubated for 30 mins at 30 ºC in 1 mL medium) were detected following separation by capillary LC-EC with gradient elution. The cp supernatant contained elevated levels of Asp, Tau, and GABA compared with the supernatant of the vz cells (2.2, 3.3 and 3.1 fold increases respectively). The level of Glu and β-Ala did not differ significantly in the two compartments, the results of which are given in Table 3-1. Since no drugs were added to the medium, these results provide evidence that amino acids are secreted by embryonic cells and suggest that *in vivo*, the cells may be creating a concentration gradient of amino acids to guide the in-growth of motoneuron terminals (cp relative to the vz cells).

In general, muscle fibers incubated over time (2 to 8 hrs) in physiological saline solution accumulates GABA, Glu and Tau to micromolar levels, Asp to sub-micromolar levels and β-Ala remains at nanomolar levels. Figure 3-2 demonstrates the increase of GABA in the supernatants generated under control conditions over time.

From the capillary LC studies, of the amino acids released, Tau showed the steepest concentration gradient between the cp and the vz, 0.62 – 2.03 μM. Since Tau is released from growth cones of developing neurons (Taylor et al., 1990), Tau was used in an in vitro chemotaxis assay to analyze the effects on dissociated cortical cells. At micromolar levels, Tau stimulated a migratory response of dissociated cells in a dose dependent manner, Figure 3-3A. Tau induces both cp and vz cells to migrate, vz cells were migrated to 1.0 μM Tau or 500 fM GABA; cp cells were migrated to 1.0 μM Tau or GABA, Figure 3-3B. These results indicate that amino acids can act as chemoattractants for cells to migrate.

Butterfly Brain Tissue

In another application, the OPA/t-BuSH method was used to measure the amine-containing neurotransmitters present in butterfly brains. Figure 3-4A illustrates a chromatogram of a butterfly brain diluted 100-fold and Figure 3-4B the same extract without dilution. In addition to the targeted neuroactive amines, other amines expected in the sample include methionine, valine, leucine, phenylalanine, ornithine, lysine, β-aminobutyric acid and α- aminobutyric acid. Unlabelled peaks observed in the chromatograms may correspond to these, but were not included in our standards and therefore not quantitated. As with the dialysate samples discussed in chapter 2, a larger dynamic range than available with this system would be required to quantify amino acids and amines in a single chromatographic run. Nevertheless, reproducible data were obtained for analysis of all the detectable amines and amino acids as shown in Table 3-2 which compares the levels found in brains of wild and naïve (i.e., housed in cages). Since neurotransmitters have been investigated to a lesser extent in insects, one can only

speculate about their function. The results indicate that neurotransmitters may have different functions in the species, and it should be possible to move further towards an understanding of the neuronal function in insects by using a combination of behavioral and control experiments.

Conclusion

The technique is ideal for analyzing small samples obtained from different biological sources. The results indicate that *in vivo*; muscle cells may release amino acids thus generating a concentration gradient that signal neurons to migrate towards the cortical regions.

Table 3-1 The levels of amino acids in supernatant of vz or cp cells from E17 cortices. Values are the mean ± SEM for 3 trials.

Amine NT	Concentration in vz (μM)	Concentration in cp (μM)
L-Asp	0.51 ± 0.18	1.10 ± 0.47
L-Glu	0.38 ± 0.09	0.53 ± 0.27
β-Ala	0.04 ± 0.02	0.03 ± 0.02
Tau	0.62 ± 0.25	2.03 ± 1.25
GABA	0.63 ± 0.21	1.97 ± 0.77

Amino acids released by embryonic cortical cells were detected by capillary LC with electrochemical detection. Results indicate that vz and cp cells spontaneously release amino acids.

Table 3-2 Neurotransmitter levels in butterfly brain samples. Five brains ground up in water were used for each analysis. Concentration of targeted neuroactive amines are reported in micromolar (μM) for Asp, Glu and GABA; nanomolar (nM) for NA, DA and 5-HT. Values are the mean ± SEM for 4 samples.

Amine NT	Wild male	Naïve male
L-Asp	80 ± 9	32 ± 5
L-Glu	100 ± 19	56 ± 7
GABA	7.8 ± 0.4	3.2 ± 0.9
NA	142 ± 21	306 ± 41
DA	14 ± 1	27 ± 4
5-HT	26 ± 1	24 ± 3

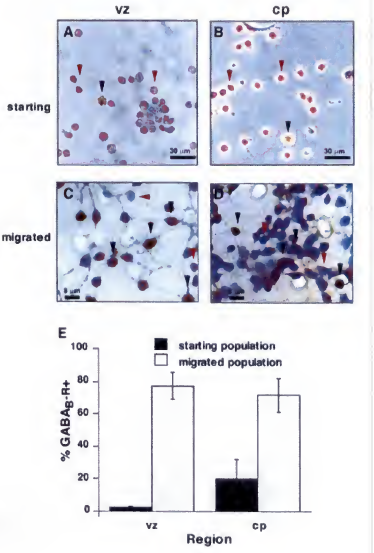


Figure 3-1 Photomicrographs of dissociated cells immunostained with anti $GABA_B$-R1 anti-serum before (A, B) or after (C, D) migration to GABA in the in vitro chemotaxis assay. Black arrows indicate $GABA_B$-R1 immunopositive. Red arrows indicate unlabelled peaks. (E) Histogram illustrating the enrichment of GAGA receptors. Source: Behar et al., 2001

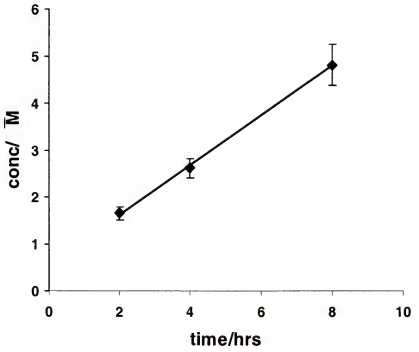


Figure 3-2 Increase in GABA levels when muscle cells were incubated in physiological saline over 8 hours.

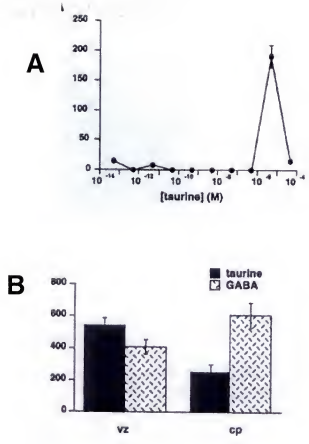


Figure 3-3 (A)Taurine induces cortical cells to migrate in vitro in a dose dependent manner. (B) Tau and GABA induce a similar number of vz cells to migrate. Source: Behar et al., 2001

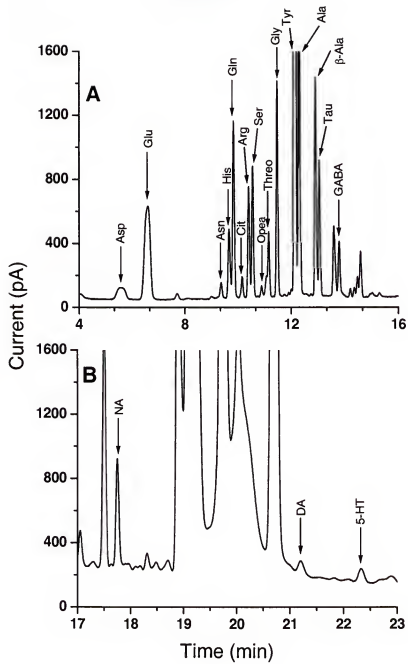


Figure 3-4 Chromatograms resulting from analysis of butterfly brain tissue samples. (A) A 1:100 dilution of sample for determination of amino acids (B) Chromatogram for detection of catecholamines and indoleamines without dilution. Peak labels are: asparagine (Asn), histidine (His), glutamine (Gln), serine (Ser), o-phenylethanolamine (o-Pea), threonine (Thr), tyrosine (Tyr), and alanine (Ala). Separation and analysis conditions are given in the text.

CHAPTER 4
OPA/THIOL DERIVATIZATION METHOD TO INCLUDE ENANTIOMERS, NON-CHIRAL AMINO ACIDS, CATECHOL AND INDOLE AMINES BY CAPILLARY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

Introduction

Until recently, it was assumed that the free amino acid pools in mammals were exclusively of the L –form (Corrigan 1969). L-amino acids have wide spread functions in the brain including neurotransmission and neuromodulation. Some of the most studied neurotransmitters are L-aspartate (Asp), L-glutamate (Glu), γ-aminobutyric acid (GABA), taurine (Tau), glycine (Gly), dopamine (DA), serotonin (5-HT), noradrenaline (NA) and histamine. L-Asp and L-Glu are the excitatory neurotransmitters responsible for normal synaptic transmission, GABA the major inhibitory neurotransmitter. However, a number of studies have provided evidence indicating that D-amino acids, such as D-Serine (Ser), D-Aspartate (Asp) and D-Alanine (Ala) present in free form or metabolically stable proteins in higher vertebrates may have important functions (Man et al., 1983; Dunlop and Niedle 1987). D-Ser occurs in the brain in glial cells localized to areas enriched in N-methyl-D-aspartate (NMDA) receptors. D-Ser appears to be an endogenous ligand for the glycine site of the NMDA receptors (Hashimoto et al., 1992; Schell et al., 1997). D-Asp is the only other D-amino acid reported to occur in significant levels in the CNS. D-Asp may play a role in the regulation of proliferation and differentiation of various tissues during development (D'Aniello et al., 1978; Hashimoto et al., 1997). Neurotransmitters are related metabolically to amino acids e.g. Glu or Asp

can be converted to GABA. Glutamine (Gln) is a precursor of Glu and consequently related to GABA and Asp, (Bradford et al., 1978; Godfrey et al., 1994). Ser is the major precursor of Gly (Aprison and Daly, 1978). To elucidate their functions, it is important to extend the current method(s) of amino acid analysis to include enantiomeric forms such as D-Asp, D-Glu, D-Ser and D-Ala.

There is increased interest in stereochemical aspects of pharmacological activity and drug design, therefore the drive to separate enantiomers. Stereoselective separation techniques are important in the pharmaceutical, chemical and agricultural industries. For example, in biological systems, enantiomers are known to produce differences in their pharmacokinetics, pharmacodynamics and metabolism. Simultaneous determination of D and L forms of amino acids in biological matrices by chromatography or electrophoresis is not trivial. Compared to general amino acid analysis, comprehensive separation implies that twice as many peaks must be resolved.

There are two approaches to the separation of enantiomers by chromatography. Enantiomers can be separated directly using chiral bonded stationary phases or by the addition of chiral additives to the mobile phase followed by separation on a non-chiral stationary phase. In the indirect method, enantiomers react with a chiral reagent forming covalently bonded diastereometric derivatives which can be separated on achiral stationary phase (Gorog et al., 1994). Labeling of amino acids improves detectability and enhances detection selectivity. For the separation of amino acid enantiomers by the indirect method, a number of chiral reagents used in pre-column derivatization have been reported. These include isothiocyanate based compounds (Dunlop et al., 1987; Peter et al., 2000), Marfey's reagent (Marfey et al., 1984), chiral monohalo-s-triazine reagents

(Bruckner et al., 1992), dansyl, o-phthaldialdehyde together with chiral thiols (Bruckner et al., 1991; Desai et al., 1993; Hashimoto et al., 1992). Since its discovery in 1971, OPA derivatives have been widely used for the determination of primary alkyl amines following reversed phase HPLC with fluorescence detection (Roth 1971). The OPA/thiol reagent reacts with primary amines, so that in general, other compounds are not detected, is readily insensitive towards matrix interferences such as high salt concentration. OPA in combination with a chiral thiol can be used for indirect enantiomeric separation on a normal column following pre-column derivatization. Examples of chiral thiols used to form diastereometric derivatives of amino acids are shown in Figure 4-1, these include N-acetyl-L-cysteine (NAC), N-acetyl-D-pencillamine (NAP), N-tert-butoxycarbonyl-L-cysteine (Boc-L-Cys) and N-isobutyryl-L-cysteine (IBC).

Experimental

Reagents and Buffers

Boric acid, OPA, amino acid and biogenic monoamine standards were obtained from Sigma (St. Louis, MO). Iodoacetamide (IAA) and N-tert-butyloxycarbonyl-L-cysteine (Boc-L-Cys) from Fluka (St. Louis, MO). Phosphate buffer salts, sodium hydroxide and ethylenediamine-tetraacetic acid (EDTA) were enzyme or ACS grade and were obtained from Fisher Scientific (Atlanta, GA). Water, acetonitrile and methanol were HPLC grade and obtained from Burdick and Jackson (Muskegon, MI).

10 mM stock solutions of neurotransmitters were prepared in 100 mM HCl containing 0.5 M ascorbic acid and diluted with artificial cerebral spinal fluid (aCSF) or water prior to use. 50 mM phosphate buffered mobile phase (containing 1mM EDTA) was made to be self adjusting at pH 6.5 by dissolving 4.22 g Na_2HPO_4 and 4.73 g NaH_2PO_4 in 1 L water. 0.6 M boric acid solution adjusted to pH 9.5 was used as the derivatization buffer.

Buffers were prepared using HPLC grade water and filtered using 0.22 μm Teflon membrane filters (Fischer Scientific, Atlanta, GA) and glass vacuum filtration system.

Derivatization Procedure

The derivatization reagent was prepared daily by dissolving 40 mg OPA and 50 mg Boc-L-Cys in 1.5 mL methanol, followed by addition of 3.5mL borate buffer (0.6 M). The solutions were stored at room temperature in darkened borosilicate glass vials that had been cleaned using 1 M HCl followed by rinses with HPLC grade water and absolute ethanol (Boyd et al., 2000). For micro scale derivatization, 0.4 μL OPA/Boc-L-Cys reagent was added using a Famos autosampler (LC Packings, San Francisco, CA) to a microvial containing 2.0 μL of the amino acid standard or dialysate sample. Derivatization was allowed to proceed for 5 minutes at room temperature. Because of the interfering peaks of the residual thiol, 0.4 μL of IAA was added immediately after the first reaction to the microvial. In this step, (a 3 minute reaction), IAA was used to scavenge the excess thiol. An aliquot (1.0 μL) of the reaction mixture was introduced into the LC- system by sandwich injection.

Capillary Liquid Chromatography

OPA derivatized free amino acid enantiomers and nonchiral amino acid neurotransmitters were analyzed by capillary LC with electrochemical detection and gradient elution using the same chromatographic system described in chapter 2. Capillary columns, 50 μm i.d. x 33 cm long fused silica capillaries (Polymicro Technologies, Phoenix AZ) were slurry packed with Alltech Adsorbsphere HS C18 – 3 μm particles (Alltech, Deerfield IL) by a previously described technique (Kennedy and Jorgenson, 1989). The eluent was delivered by two high-pressure syringe pumps (100

DM, ISCO, Lincoln, NE) operated at 1250 nL/sec (75 μL/ min). The flow rate through the columns were typically 0.9 – 1.5 nL/sec. As a result, a splitting tee was placed after the mixer to divert the majority of the mobile phase to waste. The splitter capillary used (50 μm i.d. x 30 cm long) generated a pressure of about 3700 - 3900 psi at the head of the column. Solvent A was 50 mM phosphate buffer, solvent B was a mixture of 50 mM phosphate and acetonitrile (35%/65% v/v). Mobile phase solutions were degassed prior to loading the syringe pumps by sparging with He for at least 15 minutes. Injections were performed by an autosampler (Famos) which contains a 6-port injection valve (Valco C2) fitted with a 1.0 μL injection loop. To reduce the dead volume of the system, the capillary column was threaded through the injection valve port to the rotor.

Electrochemical Detection

The working electrode was a carbon fiber microelectrode (9 μm diameter x 1 mm length) fabricated using previously described methods (Kawagoe et al., 1993). The electrode was inserted using a micropositioner into the outlet end of the capillary column mounted in an electrochemical cell containing 0.1 M KCl as supporting electrolyte. Chromatograms were obtained in amperometric mode, at an electrode potential of + 0.85 V versus Ag/AgCl reference electrodes. To improve reproducibility and reduce background current, working electrodes were pretreated by sweeping the potential from 0 to 1.8 V at a rate of 1 V/s for 30 seconds (St. Claire et al., 1985). Current was amplified using a Stanford SR-570 low noise current amplifier (Sunnyvale, CA) set at 1 Hz low pass filter. The signal was digitized using a 16-bit AT-MIO data acquisition board (National Instruments, Austin TX) in a 486 DX computer with 5 Hz collection rate.

Microdialysis

Male Sprague-Dawley rats (250 –350 g) were anesthetized with a 1.0 mL subcutaneous injection of 0.1 g/mL chloral hydrate (Sigma) and mounted in a stereotaxic frame before surgery. Probes were inserted at a rate of 500 μm/min to minimize tissue damage and were implanted at + 0.02 AP, - 0.30 ML and - 0.65 DL from bregma to sample the striatum (Paxinos and Watson, 1997). For freely moving animals, rates were anesthetized with a 0.1mL/100g body weight IM injection of a Ketamine/Xylazine cocktail (1.5 mL of 100 mg/mL Xylazine and 10 mL of 100 mg/mL). Booster shots of 0.05 mL/100g body weight were given as needed. When the animal showed no limb reflex, it was mounted in a stereotax and a guide cannula implanted into the striatum. The cannula was anchored into the skull using three screws and the cannula secured in place by application of dental cement around the cannula and screws. A cannula cap was then screwed into the cannula and the rats allowed to recover for 5- 7 days. Prior to sampling, the cannula cap was removed, the cannula flushed with saline solution and the probe implanted.

All experiments were performed in accordance with guidelines approved by the University of Florida's Institutional Animal Care and Use Committee and met criteria established by the National Institute of Health Guide for the Care and Use of laboratory Animals.

Microdialysis sampling was performed using "side-by-side" probes (3 mm x 200 μm) probes constructed in-house using methods described elsewhere (Robinson and Justice, 1991). Artificial cerebral spinal fluid (aCSF) consisted of 145 mM NaCl, 2.68 mM KCl, 1.01 mM $MgSO_4$ and 1.22 mM $CaCl_2$ (purchased from Sigma, St. Louis, MO).

For potassium stimulation, the aCSF flowing through the dialysis probe was changed to high K^+ aCSF (2.68 mM NaCl. 145 mM KCl, 1.01 mM $MgSO_4$, and 1.22 mM $CaCl_2$. aCSF or high K^+ aCSF was perfused through the probe at 0.3 µL/min using a microsyringe pump (CMA/102, Acton MA). Sample collection began after basal levels were constant, about 2 hr after probe implantation. Fractions were collected at 7-minute intervals (2.1 µL per fraction) and stored at –50 °C.

Results and Discussion

Preliminary Studies

A survey of the literature revealed that, in general, equimolar chiral thiol and OPA dissolved in MeOH followed by addition of borate buffer (pH 8-10) was used as derivatization reagent. Standard solutions were mixed with derivatization reagent, reacted for 2 - 10 minutes and injected onto a HPLC system and monitored with a fluorescence or UV detector. Columns used for chromatography are ODS 5 µm or 3 µm, separation by gradient elution using phosphate or acetate buffer as the A phase and buffer/methanol, methanol/acetonitrile, or buffer/methanol/THF as the B phase (Desai and Gal, 1993; Bruckner et al., 1994).

In this study, the resolution was optimized by varying the chiral thiol (NAC, IBC and Boc-L-Cys) in the derivatization reagent. Chromatograms of IBDC-OPA derivatives (40 mM OPA/50 mM IBC dissolved in 0.75 mL MeOH and 4.25 mL borate buffer) of standards containing enantiomers of Asp, Glu, Ser (2- 5 µM) were similar to blanks (no amines intentionally added). Columns were packed to 28 cm long with C-8 5 µm particles, gradient elution with an acetonitrile/buffer mobile phase and electrochemical detection at 0.75V. Using the same standard mixture, column, gradient and detection

potential, 3 small peaks (possibly due to the unresolved enantiomers) were observed when 50 mM NAC was used as chiral thiol. After these preliminary experiments, the use of Boc-L-Cys, a chiral thiol with a branched structure similar to t-BuSH was investigated and columns packed to 18 cm long with Allsphere ODS 3 μm particles. The electrode was poised at +0.85 V for detection after it was determined to give the optimal signal-to-noise ratio following hydrodynamic voltammograms in the range 0.4 to 1.0 V.

Figure 4-2 shows the chromatograms obtained for a blank, a mixture of D- and L-amino acids (Asp, Glu, Ser, Ala), Gly and GABA and a mixture of D and L amino acids (Asp, Glu, Ser, Gly). Mobile phase A was 50 mM phosphate buffer (pH 6.5) and mobile phase B contained 50 % acetonitrile; amino acids were eluted using a linear gradient from 30 – 70 % B in 26.7 minutes (1.5 B/min). L- and D- Asp and Glu were not retained; peaks were identified by comparison with authentic D- or L- enantiomers. Even when the standard mixture was injected in 10 %B initially and the slope of the gradient reduced to 1 B/min, Asp and Glu were not observed. Also injected on-column was a standard mixture containing targeted D-amino acids (Asp, Glu, Ser, Ala) and other amino acids (Gly, GABA, L-Ser, L-Cit, L-Ala) and a mixture containing 16 L-amino acids expected in biological matrices (Asp, Glu, Asn, Gln, His, Cit, Arg, Ser, Tyr, Ala, Thr), GABA, Gly, o-Pea, Tau and β-Ala. This is illustrated in Figure 4-3, elution was performed using a linear gradient from 10 -45 %B in 35 minutes (1.0 B/min).

Separation Conditions

To achieve retention of L- and D- Asp and Glu; better resolution for the other amines (enantiomers and non-chiral amino acids), packing material column length, strength of mobile phase and gradient steepness were investigated. The column length

was increased to 33 cm and the particles changed to Adsorbsphere HS (C18 phase with 3 μm particles but containing 20 % C load resulting in more surface area). Figure 4-4A illustrated the separation obtained for a mixture of endogenous amino acids and primary amines found in physiological samples namely, L-Asp, L-Glu, L-Asn, L-Ser, L-Gln, L-Cit, L-Thr, L-His, o-Pea, Gly, L-Arg, L-Ala, β-Ala, GABA and Tau (concentration ranging from 1-5 μM). For gradient elution, the two mobile phase system used consisted of 50 mM phosphate buffer, pH 6.5 as mobile phase A and 50 mM phosphate buffer, pH 6.5 – acetonitrile (35:65, v/v) as mobile phase B. Injection volume was typical 100 nL. The gradient profile was chosen to optimize the gradient steepness parameter (b) depending on the flow rate through the column (discussed later). Separation was carried out at 3800 psi, flow rate through the column was 1.39 nL/sec. Gradient profile was as follows, initially at 5% B for 2 minutes, linearly changed to 20 % B over 0.75 minutes (20 B/min), 30 %B over 5 minutes (2 B/min), 50 %B over 26.7 minutes (0.75 B/min). Amino acid enantiomers and other non-chiral amino acids can be separated using the same chromatographic conditions, Figure 4-4B. In all cases, the L-enantiomer elutes before the corresponding D-enantiomer. The chiral reagent used, Boc-L-Cys was optically pure (99.8%). As a result, with standard mixtures containing L-amino acids only, no D-enantiomers could be detected, Figure 4-4A.

Discussed in chapter 2, is the simultaneous determination of L- amino acids, catecholamines (NA, DA) and 5-HT following derivatization with OPA and thiol. The proceeding section(s) serves to demonstrate that the same principle can be applied to the determination of enantiomers, nonchiral amino acids, catecholamines and indoleamines within the same chromatographic run. Shown in Figure 4-5A is a representative

chromatogram of Boc-L-Cys-OPA derivatives of amino acids, NA, DA and 5-HT. Following derivatization using OPA and a chiral thiol, and separation on achiral stationary phases, enantiomers are typically detected using a fluorometer. This marks the first instance, their detection by amperometry. The diastereometric derivatives of L- and D- (Asp, Glu, Ser, Cit, Arg, Ala), Gly, GABA, NA, DA and 5-HT were separated within 50 minutes. Under the same conditions, good resolution was obtained for a standard containing 15 L-amino acids, Figure 4-5B. Gradient profile used was as follows: initially at 5 %B for 2 mins, linearly changed 25 %B over 1.3 mins (15 B/min), 40 %B over 15 mins (1 B/min), 53 %B over 17.3 mins (0.75 B/min), stepped to 98 %B, held at 98 %B for 10 mins, then stepped back to 5 %B and re-equilibrated for 50 mins, flow rate through the column was 0.97 nL/sec.

Preconcentration and Gradient Optimization

On-Column preconcentration can be used to increase concentration limits of detection (CLOD) of small sample volumes. The OPA derivatives are (i) initially injected in a weak mobile phase (one that does not allow desorption of the derivatives) and (ii) the final sample solution contains approximately 5% organic resulting in high capacity factors (k'), a condition that favors retention. Derivatives are initially stacked in a narrow zone at the head of the column for a specific time until the strength of the organic modifier is changed to allow migration along the column (as a result of decreasing k's). Chromatograms obtained (data not shown) with increasing injection volumes from 60 - 125 nL following injection of a standard mixture containing enantiomers, non-chiral amino acids, NA, DA and 5-HT demonstrate that all compounds of interest except L-and D- (Asp and Glu) preconcentrate well on-column. This is further illustrated by comparing plots of peak height versus injection volume, Figure 4-6 for D-

Glu ($r^2 = 0.384$), D-Ser, GABA and NA (the peak height is increased linearly, $r^2 > 0.988$, peak areas were also increased). As a result, an injection volume of 100 nL was used throughout this work. The gradient was optimized using the Linear Solvent Strength (LSS) theory (Snyder et al., 1979). For example, with the column used in Figure 4-5, b = 0.125, resulting in k" of 6.944 which is optimal, range of b and k" should fall between 0.43 – 0.087 and 2-10 respectively.

Reproducibility and Detection Limits

The linearity of the concentration versus response relationship was established over the range 0.1 -10 μM for each of the amine neurotransmitter. Linear regression analysis from the calibration graphs indicated that the correlation coefficient ranged between 0.9755- 0.9997 and 0.9866-1 for peak height and peak area respectively. The relative standard deviation of retention times, peak heights and peak areas of a L- and D- amino acid standard (1000 nM) derivatized with OPA and Boc-L-Cys (performed in triplicate) is shown in Figure 4-7. The detection limits were determined for the OPA-Boc-L-Cys derivative of chiral and non-chiral neuroactive amines. Detection limits were calculated as the amount required to give a signal-to-noise ratio of 2, calculated using a p-p noise of 2 pA and 9 pA for the amino acids and (NA, DA and 5-HT) respectively. Value listed in Table 4-1 as CLOD and MLOD are an order of magnitude greater than the minimum detectable quantity obtained using t-BuSH for the same amino acids (chapter 2). This can be attributed to a larger injection volume of 250 nL, a lower p-p noise for the OPA/t-BuSH derivatives.

In Vivo Basal release of D-amino acids

Figure 4-8 shows representative chromatograms of Boc-L-Cys derivatives of a blank, standard (amino acid enantiomers, non-chiral amino acids, NA, DA and 5-HT) and

dialysate obtained from the striatum of an anesthetized rat. Mobile phase A was 50 mM phosphate buffer, pH6.5 and mobile phase B was the phosphate buffer with 65 % acetonitrile. The separation was carried out at 3800 psi, 0.87 nL/sec using a column (33 cm x 50 μm i.d) packed with Adsorbsphere HS C-18 3 μm particles. Gradient elution started initially with 5 % B for 2 minutes, linearly changed to 28 % B at 20 B/min, to 40 % B at 0.75 B/min, to 50 % B at 1B/min then to 98 % B at 10 B/min. The basal dialysate concentration and *in vitro* probe recoveries of L-Asp, D-Asp, L-Glu, D-Glu, D-Ser, Gly, GABA and NA are given in Table 4-2. The basal levels of L-amino acids and non-chiral amino acids in the striatum of anesthetized animals in this study were consistent with those obtained previously using OPA and t-BuSH (chapter 2). Figure 4-9 compares the estimated extracellular levels of L-Asp, L-Glu, GABA and NA following correction for *in vitro* recovery in both methods. The only slight discrepancy is for GABA. Indeed GABA levels obtained by the present method are somewhat higher, probably due to errors in quantitation because of its close proximity to D-Ala and Tau. For the additional amino acids monitored in this study and in particular D-Asp, D-Glu, D-Ser, dialysate concentration in the striatum was 40 nM, 129 nM and 4.81 μM respectively. Of these, D-Ser is the most studied and its extracellular level in the striatum of rats is estimated to be approximately 2.5 times that of Gly (Hashimoto et al., 1995). If the results given in Table 4-2 are corrected for *in vitro* probe recovery, D-Ser is approximately 2.2 times that of Gly and thus consistent with the findings of Hashimoto. The level of D- Ser in the rat brain are estimated to be one third (1/3) those of L-Ser (Hashimoto et al., 1995). D-Ala and D-Asp is present in brain tissues at very low levels (Hashimoto and Oka, 1997). For example, in the mammalian brain, D-Asp is estimated to be 100 fold less than L-Glu or

L-Asp (D'Aniello et al., 1998). In another report, D-Asp level in mouse pineal gland is one-third (1/3) that of L-Asp (Schell et al., 1997). D-Ala levels in mouse brain tissues is approximately 8 fold lower than D-Asp (Hamase et al., 1997; Morikawa et al., 2001). In light of this our detection limit for D-Ala would not permit the detection of D-Ala. The basal level of DA could not be determined in all cases due to an increase in baseline drift with increase in organic content of mobile phase. This limits the method for the simultaneous monitoring of amino acids, catecholamines and indoleamines.

Response to Potassium Stimulation

Activation of synaptic neurotransmitter release from brain regions can be achieved by exposure to high potassium (K^+) which leads to depolarization of neurons and glial (Zheng et al., 2000). The effect of amino acid release from an individual animal to depolarization by potassium stimulation is shown in Figure 4-8 D, which illustrates successive chromatograms of a dialysate sample before and after stimulation with K^+. The magnitude of the release is summarized in the bar-graph in Figure 4-10. Perfusion with 150 mM K^+ for 10 mins results in increases in the dialysate concentration of L-Asp, L-Glu, Gly, GABA, Tau, NA while a decrease was observed for D-Glu, L-Ser, D-Ser and L-Gln. The release pattern for the L- amino acids was similar to those reported (Zheng et al., 2000; Boyd et al, 2000; Morales-Villagran and Tapia, 1996,). The effect of potassium stimulation on D-amino acid release is not well understood as there have only been few reports. For example, potassium stimulation of the rabbit cortex led to a slight decrease (not statistically significant) in the level of D-Ser (Lo et al., 1998), while an increase in D-Asp was observed following potassium injection into the cerebral cortex of mice (Schell et al., 1997). Depolarization induced by potassium stimulation is shown to produce increases in catecholamine release from the striatum (Aliaga et al., 1995;

Gerhardt and Maloney, 1999). In this work, perfusion with 150 mM K^+ for 10 minutes resulted in an increase in basal level of NA.

Anesthetized vs. Awake Animals

In vivo procedures involve anesthetizing the animal prior to implantation of the probe for microdialysis. The use of general anesthesia is shown to alter the release and reuptake of neurotransmitters as well as responsiveness of neurons to different activities (Kiyatkin and Rebec, 1996; Kelland et al., 1989; Hamilton et al., 1992). Today, microdialysis in awake animals has become firmly established as a versatile and reliable *in vivo* method to monitor neurotransmitter release (Westerink, 1995; Young, 1994). There is an obvious difference in the chromatograms obtained for basal activity of an anesthetized and a free-moving rat, Figure 4-8. The responsiveness of various neurotransmitters is altered by the use of general anesthesia as illustrated in Figure 4-11. For example, the basal level of DA in an anesthetized animal could not be reliably established, however in an awake animal, the levels are not suppressed, Figure 4-12. In this way, the use of awake animals should reduce the demand on sensitivity of the method and provide a more accurate account when studying neurotransmitter dynamics. This study agrees with the finding that the use of general anesthesia may influence the result and the interpretation of neurotransmitter dynamics within the brain (Ungerstedt 1991).

At a flow rate of 2 μL/min, the basal dialysate concentration for D-Ser in the striatum of free-moving rats is reported to be 7.3 μM (Hashimoto et al, 1995). From Figure 4-8D, the concentration of D-Ser in the dialysate of a free-moving rat is estimated to be 8.6 ±

0.85 μM at a flow rate of 0.3 μL/min. In both cases, the concentration has been adjusted to assume a probe recovery of 100.

Faster Separations

Even though the advantages of simultaneous determination of different classes of neurotransmitters in microdialysis samples are clearly demonstrated, there are some draw backs. Due to the complex nature of neurotransmitter systems, a large number of samples are generally analyzed. A comprehensive analysis of amino acid enantiomers, non-chiral amino acid, NA, DA and 5-HT requires a separation time of 45 minutes in addition to a column re-equilibration of 40-50 minutes. As a result, few samples can be analyzed in any given day. The through-put of the system can be increased by a reduction in separation time through the use of shorter columns packed with smaller particles. To illustrate this Alltima C-8 3 μm particles were used to pack columns to a length of 15 cm and used to separate a mixture containing L-Asp, L-Glu, L-Cit, L-Arg, Gly, Tau, GABA, DA and 5-HT following derivatization with OPA and t-BuSH. As seen in Figure 4-13, a reduction in separation time by approximately one-half is achieved. It should be noted that the gradient used for the separation used in Figure 4-13 was not optimized; therefore, through gradient optimization separation time could be decreased further. The use of smaller particles has become a popular way to reduce analysis time (MacNair et al., 1999). In principle, the same theory can be applied to the work in this chapter, that is, the use of shorter column packed with 1.5μm particles.

Conclusion

The electrochemical properties of chiral OPA derivatives have not been previously explored. In this work, the simultaneous detection and quantitation of chiral

amino acids such as Asp, Glu, Ser, non-chiral amino acids such as Gly, Tau, β-Ala, catecholamines and indoleamines (NA, DA, 5-HT) with electrochemical detection is presented. The method requires minimum sample preparation in comparison to GC, ion-exchange or TLC. The results have confirmed the existence of free D-Ser, D-Asp in the rat brain and is shown to be suitable for the determination of amino acids enantiomers. The reliability is supported by comparing the levels of L-amino acids and non-chiral amino acids in the rat brain obtained in this study and those previously obtained (chapter 2). A disadvantage however is the long analysis time, in part due to the time required to re-equilibrate the column following gradient elution.

Table 4-1 Limits of detection of amine neurotransmitters.

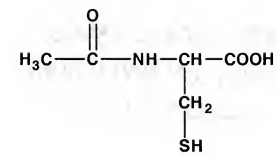
Amine NT	CLOD (nM)	MLOD (fmoles)
L-Asp	27	3
D-Asp	23	2
L-Glu	30	3
D-Glu	23	2
L-Ser	6	.6
D-Ser	2	.2
L-Cit	10	1
Gly	19	2
D-Ala	27	3
GABA	30	3
NA	18	2
DA	30	3
5-HT	142	14

Calculated as the concentration corresponding to 2 x S/N. Evaluated using three consecutive analyses of a standard mixture (1 μM) and injection volume of 100 nL.

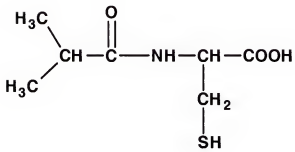
Table 4-2 Basal concentration and *in vitro* probe recovery of amine neurotransmitters in the striatum of anesthetized rats.

Amine NT	Basal concentration (μM)	In Vitro Probe Recovery (%)
L-Asp	.24 ± .02	61 ± 17
D-Asp	.04 ± .01	58 ± 9
L-Glu	.55 ± .15	58 ± 3
D-Glu	.13 ± .02	56 ± 5
D-Ser	4.8 ± .47	74 ± 9
Gly	1.7 ± .04	58 ± 16
GABA	.30 ± .05	68 ± 3
NA	.05 ± .01	92 ± 16

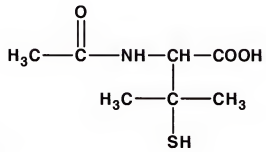
Errors are the standard errors of the mean. Five rats were used to determine basal levels. *In vivo* dialysate concentrations have not been corrected for *in vitro* probe recovery.



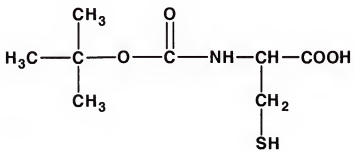
NAC



IBC



NAP



Boc-Cys

Figure 4-1 The chemical structures of different chiral thiols used in combination with OPA for chiral derivatization of amino acids.

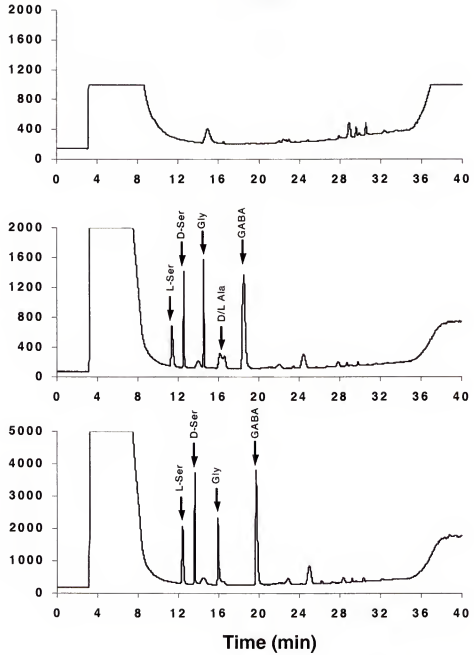


Figure 4-2 Chromatograms of Boc-L-Cys- OPA derivatives of a blank (top trace), standard mixture containing L-and D- (Asp, Glu, Ser, Ala), Gly, GABA and a mixture containing L- and D- (Asp, Glu, Ser), Gly, GABA (bottom trace). Column 18 cm x 50 µm id, Allsphere ODS, 3 µm. Gradient 30 – 70 %B at 1.5 B/min, standards each at a concentration of 10 µM.

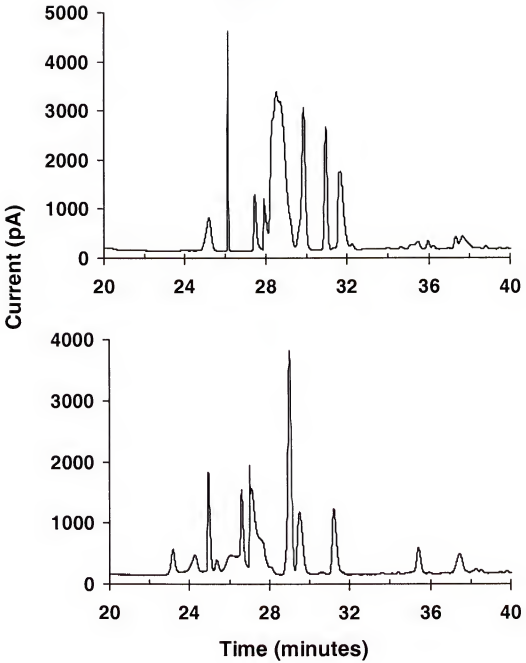


Figure 4-3 Chromatograms of Boc-L-Cys- OPA derivatives of a L-and D- amino acids (top trace), 16 L-amino acids (bottom trace). Column 18 cm x 50 μm id, Allsphere ODS, 3 μm. Gradient 10 – 45 %B at 1.0 B/min, concentrations in the range 2- 10 μM. From comparison, with authentic amino acids, L- and D- Asp and Glu are non-retained.

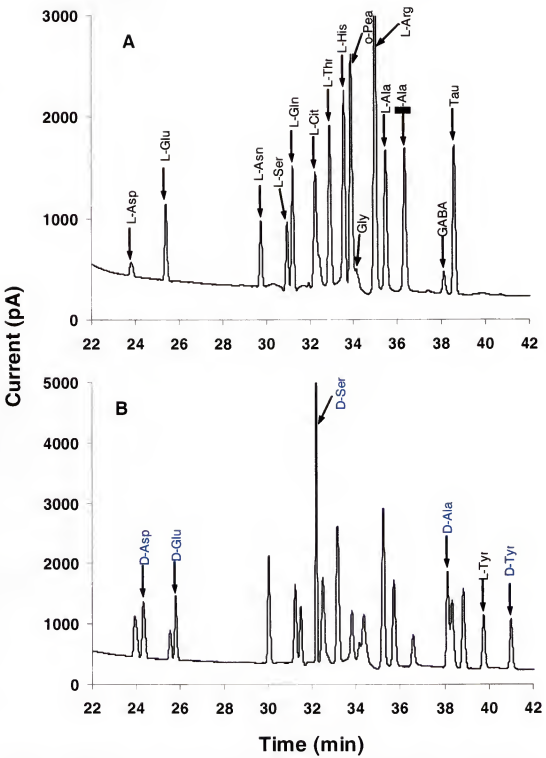


Figure 4-4 Separation of amino acid enantiomers and non-chiral amino acids using columns (50 µm i.d x 33 cm) packed with Adsorbsphere HS C-18 3 µm particles at 3800 psi, 1.4 nL/sec. Chromatograms are as follows (A) a standard 16-component mixture of L-amino acids and (B) 16 L-amino acid (same as in A) and D-amino acids (Asp, Glu, Ser, Ala Tyr).

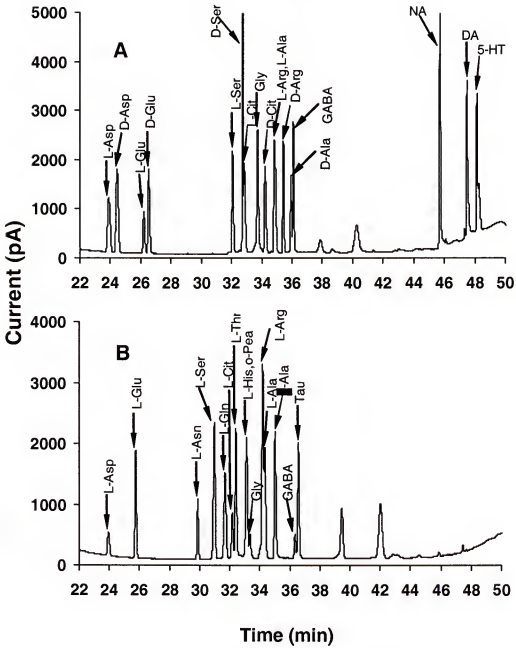


Figure 4-5 Chromatograms of (A) amino acid enantiomers, nonchiral amino acids, NA, DA and 5-HT. (B) L-amino acids only. Concentration range 1-5 μM. Separation was carried out using a 50μm i.d x 36 cm long column packed with Adsorbsphere HS C-18 3 μm particles. Flow rate through the column, 0.97 nL/sec.

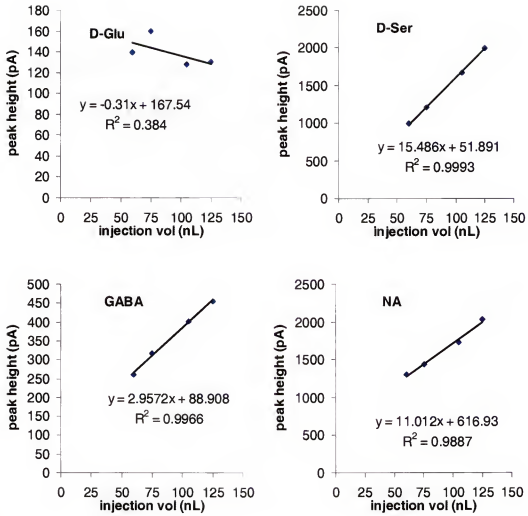


Figure 4-6 Plots of peak height versus injection volume to illustrate the inability of the first eluting amino acids (D-Glu shown) to preconcentrate on-column.

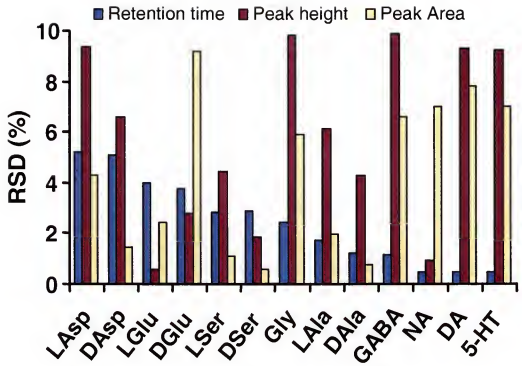


Figure 4-7 Reproducibility of retention time, peak height and peak area. Measurements were performed in triplicate of standards containing L- and D- amino acids, non chiral amino acids, NA, DA and 5-HT (each at a concentration of 1 μM) following derivatization with OPA and Boc-L-Cys.

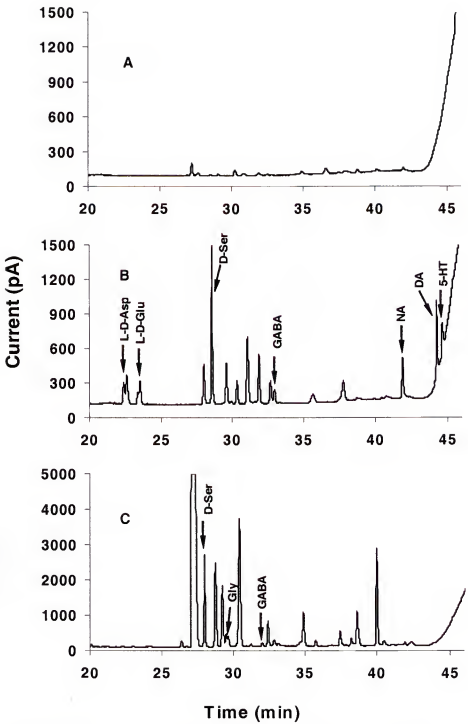


Figure 4-8 Chromatograms of Boc-L-Cys-OPA derivatives of (A) blank (B) standard mixture of amino acid enantiomers, non-chiral amino acids, NA, DA and 5-HT, amines at 1- 2.5 μM (C) dialysate obtained from the striatum of anesthetized rat following perfusion of aCSF at 0.3 μL/min through the probe. For chromatographic conditions and basal concentrations see Experimental and Table 4-2 respectively.

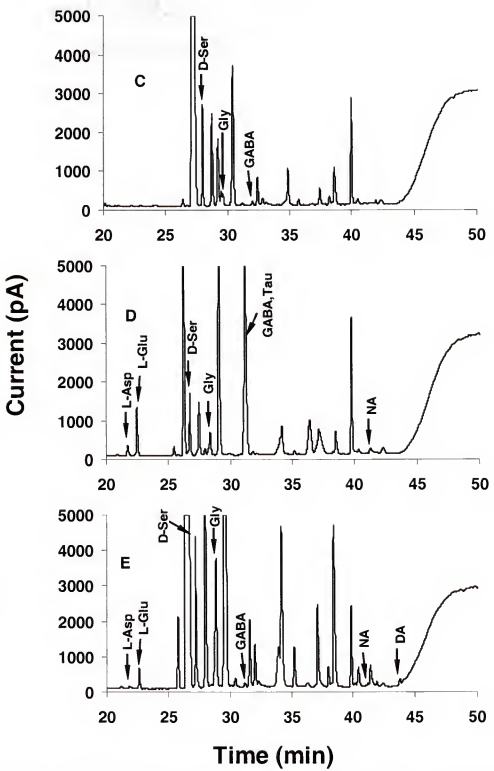


Figure 4-8 Chromatograms of (C) basal dialysate obtained from the striatum of an anesthetized rat. (D) dialysate obtained from the striatum of an anesthetized rat following potassium stimulation (E) basal dialysate obtained from the striatum of a free moving rat. The dialysis probe was perfused at a rate of 0.3 μL/min, samples collected at 7 minute interval, derivatized with OPA and Boc-L-Cys. Chromatographic conditions are given in the text.

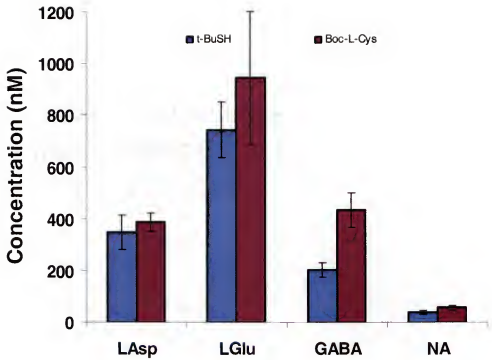


Figure 4-9 Concentration of amino acids obtained from the striatum of anesthetized rats by two methods based on OPA and thiol derivatization.

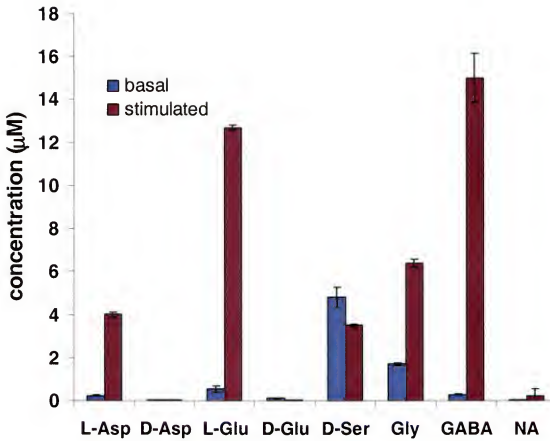


Figure 4-10 Effect of potassium stimulation on the basal extracellular concentration of L-Asp, D-Asp, L-Glu, D-Glu, D-Ser, Gly, GABA and NA in the striatum of anesthetized rats. The probe was perfused with a-CSF and a-CSF supplemented with high potassium (150 mM) at 0.3 μL and fractions collected at 7 min intervals. Results are averages with SEM of data obtained from five animals and have not been corrected for recovery.

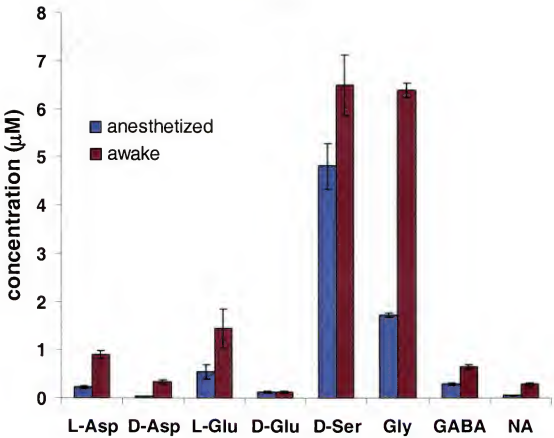


Figure 4-11 The effects of anesthesia on the responsiveness of basal L-Asp, D-Asp, L-Glu, D-Glu, D-Ser, Gly, GABA and NA as compared to the response in free-moving rats. The difference in concentration under the experimental conditions approach statistical significance.

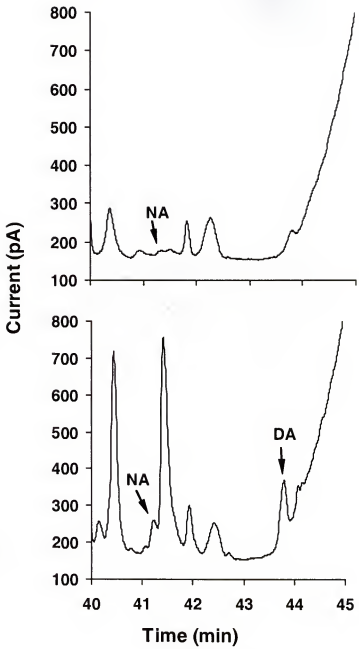


Figure 4-12 Chromatograms to illustrate a reduction in the demand on sensitivity. Top trace represents dialysate from an anesthetized rat, bottom trace dialysate from a free moving rat. Probes were implanted in the striatum and perfused at 0.3 μL/min, perfusates collected at 7 minute intervals, derivatized using OPA and Boc-L-Cys and chromatographed.

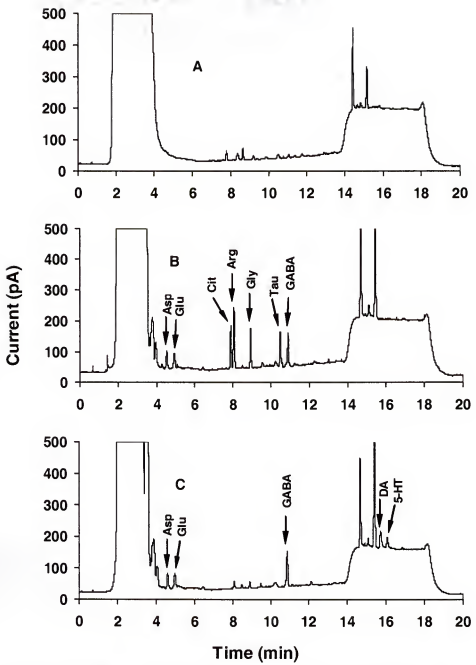


Figure 4-13 Faster separations using smaller particles (Alltima C-8 3μm) for OPA/tBuSH derivatives. Column length, 15 cm, flow rate 3.4 nL/sec, injection volume 250 nL. Gradient: initial 35% MPB increasing to 65% at a rate of 2% B/minute. A: blank. B: standard containing amino acids at 50 nM. C: standard containing L-Asp, L-Glu, GABA, DA and 5-HT each at 50 nM.

CHAPTER 5
METHOD VALIDATION- D-AMINO ACID ANALYSIS APPLIED TO NEURONS FROM APLYSIA, LIMITATIONS OF THE METHOD

One important goal of the neuroscience community is to relate the activity and functions of neurons to behavior; however, the complexity of the mammalian central nervous system (CNS) makes this a difficult and challenging task. The simpler nervous system of molluscs consisting of approximately 10,000 neurons contained in groups (ganglia) provides a useful model for studying the modulation of behavior. Chemical molecules including small molecule neurotransmitters and neuropeptides released from specific neurons are known to play a role in the mechanisms of learning and memory (Bailey et al., 2000; Craig and Boudin, 2001).

The neurons from the CNS of marine snails, Aplysia Californica are relatively large, easily identified based on location, size and are easily isolated by microdissection (Kaldany et al., 1985; Brezina and Weiss, 1997). The work presented in this chapter aims to validate the method developed in chapter 4. The levels of D-amino acids, in particular D-Asp and D-Glu detected in the striatum of the rat brain were in general low. Ganglia from mollusks were chosen as test samples to determine if any, the levels of D-amino acid present in the ganglia. Neurotransmitter tend to be present at high levels, for example, intracellular concentrations of L-Arg and L-Cit measured as markers for nitric oxide in Aplysia Californica was reported to be in the range 1 μM – 6 mM (Floyd et al., 1998), Asp in single identified neurons varied between 22 to 96 mM (Zeman et al., 1975).

Experimental

Animal and Cell Preparation

Aplysia Californica (100- 250 g) were obtained from the Aplysia Research Facility (Miami, FL) and Marinus (Long Beach, CA). They were kept in an aquarium containing continuously circulating and filtered sea water at 14-15 °C. Animals were anesthetized by injection (50% of body weight) of isotonic $MgCl_2$ (337 mM) into the body cavity. Individual ganglia were dissected (cerebral, left pedal, right pedal, right pleural and abdomen), out. The ganglionic connective tissue was removed, the nervous system sheath removed by incubating the ganglia in ASW (460 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 11 mm $CaCl_2$ and 10 mM HEPES buffer) containing 1% protease at 36 °C for 45-90 minutes depending on animal size. The ganglia were washed in ASW, whole ganglia were placed in microvials, 10 μL distilled water added and the samples stored at -50°C until analyzed by capillary LC-EC. The type and size of the individual ganglia used in this work are given in Table 5-1.

Table 5-1 The type and size of ganglia analyzed in this study.

Ganglia	Size (μm)
Cerebral	1750 x 750 x 600
Left Pedal	1300 x 800 x 800
Right Pedal	1300 x 800 x 800
Right Pleural	850 x 800 x 700
Abdominal	1620 x 1250 x 800

Before analysis, the samples were homogenized for 2 minutes using a sealed glass capillary, diluted by the by the addition of HPLC grade water (10 μL), sonicated (10 minutes) and centrifuged (27°C, 2000 rpm, 5 minutes). The supernatant was removed,

diluted 1/10 followed by a second 1/10 dilution. A small portion of the diluted sample was derivatized and injected onto the capillary LC system.

Homogenate Analysis

Standards of free amino acid enantiomers and nonchiral amino acid neurotransmitters and homogenates were derivatized by reacting with OPA and Boc-L-Cys (detailed in chapter 4). OPA derivatives were analyzed by capillary LC with electrochemical detection and gradient elution using a chromatographic system similar to the one described in chapter 2 except the ISCO pumps were replaced by an Agilent 1100 series binary pump. The high pressure ISCO syringe pumps developed leaks easily and had to be disassembled weekly for cleaning or seals replacement. This did not eliminate the problem, so to further to ameliorate the problem; the flow rate was increased from 75 to 150 µL/min, flow rate through the column, 1.4 nL/sec. Since each analysis was approximately 1 hour 45 minutes (to achieve complete resolution of amino acid enantiomers, non-chiral amino acids, NA, DA and 5-HT), this meant that in any given day, only about 5 samples (blank, standard, biological sample) could be analyzed before the syringe pumps had to be re-filled (maximum capacity of 100 mL).

Capillary columns, 50 µm i.d. x 33 cm long fused silica capillaries (Polymicro Technologies, Phoenix AZ) were slurry packed with Alltech Adsorbsphere HS C18 – 3 µm particles (Alltech, Deerfield IL) by a previously described technique (Kennedy et al., 1989). The mobile phase was delivered at 500 µL/min using the Agilent pump. Solvent A was 50 mM phosphate buffer, solvent B was a mixture of 50 mM phosphate and acetonitrile (35%/65% v/v). Mobile phase solutions were degassed by constantly sparging with He. Boc-L-Cys-OPA derivatives were separated by gradient elution and

detected by amperometry using a carbon-fiber micro-electrode (poised at 0.85 V) placed in the outlet of the capillary. Gradient profile was as follows, initially at 5% B for 1.5 minutes, linearly changed to 25 %B at a rate of 5 B/min, 25-40 %B at a rate of 1 B/min, 40-50 %B at a rate of 2 B/min, 50-98 %B at a rate of 10 B/min, held at 98 % B for 5 minutes, stepped to 5 %B over 30 seconds, re-equilibrated for 50 minutes.

Results and Discussion

Using the Agilent pump, the RSD of retention (n=3) for all analytes in the standard mixture was significantly improved (< 2%) compared to that previously reported in chapter 4 with the ISCO syringe pumps. Figure 5.1 depicts the separation of a standard containing enantiomeric mixtures of Asp, Glu, Ser and Ala, L-Cit, L-Arg, Gly GABA, NA, DA and 5-HTa diluted sample from the left pedal ganglia (volumes of each ganglia were calculated from measurements of the diameter of each ganglia made at the time of dissection). Sections of chromatograms where L- and D- (Asp and Glu), NA, DA and 5-HT elute are represented in Figures 5.2 and 5-3 respectively. Many other amine-containing neurotransmitters, present in the sample were not included in the Figures to improve clarity. These include L-Asn, L-Gln, L-Ser, L-Cit, L-Arg, GABA, Tau, Gly etc. Enantiomers are identified by correlating retention times with standards. For the sample (right pedal ganglia) depicted in Figure 5-2B at a dilution of 1/6200, peaks matching the migration time of the standard are assigned to L-Asp, D-Asp and L-Glu. In general, it was necessary to chromatograph a sample diluted 1/620 inorder to identify D-Glu, Figure 5-2C. Shown in Figure 5-3 is the section of the chromatogram where NA, DA and 5-HT elute. Data from the representative neurons used in this study are presented in Table 5-2.

Table 5-2 Results for five Aplysia ganglia assayed for L- and D-amino acids, NA, DA and 5-HT.

	Cerebral	Left Pedal	Right Pedal	Right Pleural	Abdominal
L-Asp	50	50	142	40	37
D-Asp	6.2	.50	12	3.0	4.8
L-Glu	37	37	124	30	24
D-Glu	.06	.37	.31	.10	.32
NA	25	.62	25	.60	.13
DA	.62	.31	.31	ND	ND
5-HT	1.9	.50	.43	ND	ND

Values in Table indicate concentration (mM). Results are corrected for dilution (based on cell volume). ND indicates not detected at a dilution of 1/1000 and 1/160 for right pleural and abdominal respectively.

There are limited reports related to the concentration of neurotransmitters, in molluscs, however it has been reported that the intracellular concentration of 5-HT in selected molluskan neurons is in the range 0.2-1mM (Ono and McCaman, 1984). In another report, 5-HT was reported to be present at 380 μM (Fuller et al., 1998). In this work, 5-HT present in the whole ganglia is the range 430 – 1900 μM. In one of the ganglia, a peak is observed in the chromatogram that matches the retention time of D-Ser in the standard. However, since D-Ser is thought to occur in the brain in glial cells rather than neurons and localized to areas enriched in N-methyl-D-aspartate (NMDA) receptors, it is doubtful that D-Ser is present (Snyder and Ferris, 2000; Wolosker et al., 2000).

Amine-containing signaling molecules can be identified with this technique and the contents of different ganglia compared. For example, the analysis of two similar ganglia, left and right pedal of similar size appear different their assay. The same amine-

containing neurotransmitters appear to be present in both, but the two types contained large differences in the concentration of neurotransmitters. These differences are interesting for comparative physiological studies.

Conclusion

Simultaneous detection and quantitation of small signaling molecules in single neurons provide background and fundamental information for cell physiology. An individual neuron contains many different neurotransmitters; adjacent cells often have different neurotransmitters at different level. As a result, pooled samples may not provide an accurate reflection of cell physiology. Based on the diameter of the individual neurons within the ganglia, and the detection limit, estimates of endogenous concentrations of different neurotransmitters can be made. Some neurotransmitters were not detected (Table 5-2), because the appropriate dilution were not made.

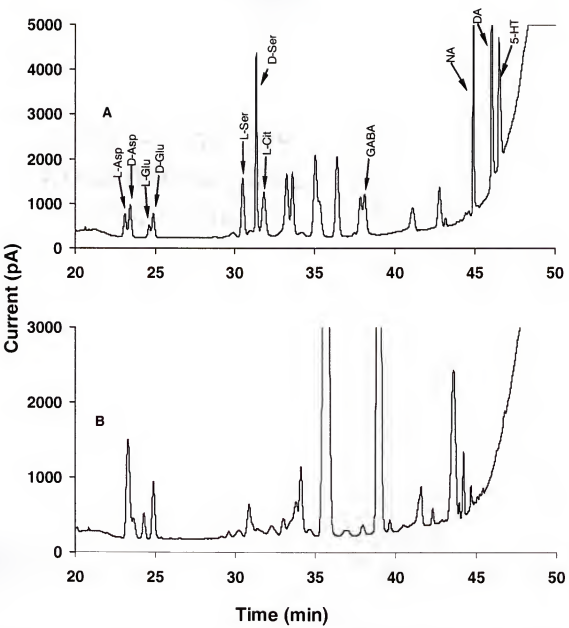


Figure 5-1 Chromatograms of A: standard containing amino acid enantiomers, non-chiral amino acids, NA, DA and 5-HT. B: homogenate from the left pedal ganglia following a 1/620 dilution.

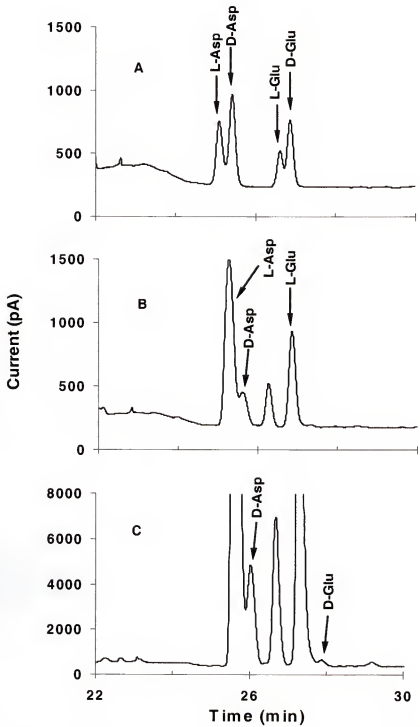


Figure 5-2 Chromatogram illustrating the portion where Asp and Glu elute (many additional neurotransmitters are detectable but are not included to improve clarity). (A) standard (B) homogenate from a ganglia diluted 1/6200 (C) same as B, but diluted 1/620.

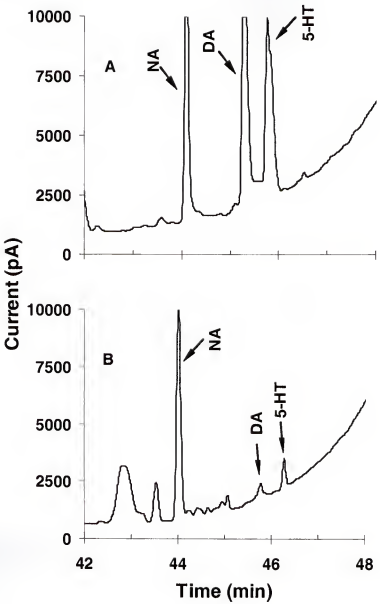


Figure 5-3 Chromatogram illustrating the portion where NA, DA and 5-HT (many additional neurotransmitters are detectable but are not included to improve clarity). (A) standard (B) homogenate from a ganglia diluted 1/600.

CHAPTER 6
SUMMARY AND FUTURE DIRECTION

Any given brain region contains several neurotransmitters, and it is likely that neurochemical changes *in vivo* results from the interaction of all released neurotransmitters. Following microdialysis, a variety of neurotransmitters and related metabolites are recovered (Robinson and Justice, 1991). However, traditional approaches to microdialysis sample analysis are focused on the monitoring of only one (or one class of) neurotransmitter(s) following a physiological or pharmacological manipulation. In general, a large number of samples containing low levels of neurotransmitters are obtained following *in vivo* experiments. The method of quantitation for analyzing such samples must be highly selective, fast, automated, capable of simultaneous measurement and have high sensitivity.

The general applicability of the OPA/ thiol pre-column derivation method to monitor L-and D-amino acids, nonchiral amino acids, NA, DA and 5-HT simultaneously was demonstrated in various areas of biosciences. Investigated were higher mammals, rats (dialysate and tissue samples), invertebrates (butterflies and snails).

A method based on OPA derivatization using t-BuSH was developed. The method offers high sensitivity and fairly rapid separations for a large number of compounds (16 L-amino acids, NA, DA, histamine and 5-HT) while requiring small sample and injection volumes (250 nL injected on-column). The applicability of the method was demonstrated to monitor amine-containing neurotransmitters following *in vivo* microdialysis of the rat

striatum. Demonstrated for the first time, was the simultaneous monitoring of different classes of neurotransmitters within the same chromatographic run. The method illustrated that more data is obtained from a single dialysate sample as many compound (representing different classes) were analyzed in the same run without sample splitting.

The application of the OPA/t-BuSH method to monitor neurotransmitters from different biological sources was presented. Neurotransmitters have been studied to a considerably lesser extent in insects; however, the different distribution of neurotransmitters in wild and naïve butterflies may indicate differences in function or behavior. Working in conjunction with others at NIH, GABA was shown to play a role in the CNS during embryonic development. In general, muscle fibers incubated over time (2 to 8 hrs) in physiological saline solution accumulate GABA, Glu and Tau to micromolar levels, Asp to sub-micromolar levels and β-Ala remains at nanomolar levels.

Until recently, D-amino acids have been thought to occur only in bacteria, but have been demonstrated in mammalian tissue. The method was extended to include the analysis of D-amino acids; that is, the simultaneous determination of chiral and non-chiral amino acid, NA, DA and 5-HT. Even though the limits of detection for the L-amino acids were a bit worse than those previously reported, D-amino acids could be detected in dialysate samples. The results obtained for L-amino acids, NA, DA and 5-HT when monitored following derivatization using t-BuSH and Boc-L-Cys were comparable. Even though the detection of OPA/Boc-L-Cys derivatives by fluorescence, have been widely explored, this marks the first instance in which electrochemical detection has been used to quantitate the derivatives.

D-amino acid analysis following derivatization with OPA and Boc-L-Cys was further validated using neurons from Aplysia Californica. The simpler nervous system of molluscs was chosen because the neurons are bigger compared to mammals and therefore the levels of amino acids are higher. D-amino acids are present at micromolar levels.

Even though the advantages of simultaneous determination of different classes of neurotransmitters in microdialysis samples are clearly demonstrated, there are some draw-backs. The simultaneous determination of several classes of compounds with a wide range of concentration (low nanomolar to micromolar levels) within a single analysis required the combination of a low flow rate (0.3 μL/min) and a relatively long collection time (7 minute). High temporal resolution is important because an accurate reflection of changes in the extracellular concentration is desired to fully understand neurotransmitter activity. As a result, there is partial loss in temporal pattern due to rapid fluctuations in neurotransmitter levels.

Since the derivatization reaction with OPA is selective for primary amines, a large number of other constituents in the brain could be potentially identified as evidenced by the number of unidentified peaks (which did not correspond to peaks in the standards) in the chromatograms. The main limitation of the method is the lack of dynamic range to cover large differences in concentration typically found for amino acids and amines in brain samples. The trace levels of NA and DA found in dialysates were quantitated; however, other neuroactive amines such as Arg, Tau and Gly were off-scale at the sensitivity required to detect NA and DA at low nanomolar level. This problem can be readily solved by automatic gain ranging as the analytes elute from the column, or the use of multiple channels operating at different sensitivities. The method presented is not

capable of monitoring all the neurotransmitters of interest; for example, 5-HT and D-Ala present in the brain are typically not detected by this method. OPA derivatives of primary amines are both electroactive and fluorescent. In some cases, depending on the thiol used, the signal obtained when measured by fluorescence is twice that of the electrochemistry. It would be interesting to couple the capillary LC system with a fluorometer to quantitate the levels by capillary LC-LIF. In general, extracellular neurotransmitter measured following microdialysis was about 30-45 % higher in awake animals. With the use of awake animals and multiple channels for detection, the biological roles and interactions between neurotransmitters in the brain can be clearly revealed.

APPENDIX A
PREPARATION OF PACKED CAPILLARY REVERSED PHASE COLUMNS

Capillary Columns (360 μm o.d. x 50 μm i.d.) were packed in-house using commercially available bulk material. The ends of capillary columns 40-cm in length were briefly heated with a flame to remove 1cm of polyimide coating. A temporary end frit was first made by tapping one end of the capillary into a conical microvial containing 15.5 μm borosilicate glass beads. The glass beads were sintered by quickly passing the end of the capillary through the cool region of a flame.

Borosilicate glass beads (7.8 μm) were used to make a slurry (10mg/mL 2-propanol) which was sonicated for 1 minute before placing in a gas pressurized high pressure bomb (400 psi). The inlet of the capillary was placed into the center of the bomb reservoir containing the slurry. The gas pressure was turned on for about 20 seconds, during which a small portion (1-cm) of the column was packed. The bomb was then de-pressurized, the inlet removed from the slurry, but remained inside the bomb and the pressure re-applied. Re-application of pressure resulted in (i) a packed region of about 2-3 cm because all glass beads present inside the column were transported towards the outlet (ii) a dried packed bed.

The outlet of the column with the inlet still held in the center of the bomb was positioned between the two electrodes of a micro-arc. The column was manipulated so that only the very end of the packed bed was positioned between the electrodes. The final frit was sintered by 'zapping' the capillary until the polyimide was charred from the capillary. The temporary end frit is then cut; the bomb pressurized which results in the

removal of un-sintered glass beads. This resulted in column blanks with recessed frits (0.05 – 0.1 mm in length) necessary for in-column electrochemical detection. In some cases, unsintered glass remaining in the column could prevent electrode placement and are removed by placing the outlet of the column in a beaker containing water and sonicated. Undesirable frits leading to decreased column efficiencies and increased back pressure can be obtained if the following conditions are not controlled during the sintering process. Factors to be considered include the arc time, current of arc, dryness of the bed and to a lesser extent whether the bomb is pressurized. This is because long arc times and a high current will result in 'over sintering' producing frits that are too tight. Partially wet beads will tend to scatter during sintering. In addition, if the bed is not completely dried, after sintering the remaining beads (1-2 cm) in the column will be transported to the outlet resulting in decreased column efficiencies.

The column blanks (40 cm) were slurry packed using commercially available bulk material by previously described techniques (Kennedy et al., 1989). Slurry of packing material (10mg/mL acetone) was sonicated and placed in the reservoir of a high-pressure packing bomb. Columns (38 cm long) containing 5 μm or 3 μm particles were generally packed at 4000 psi using a Pneumatic Amplifier Pump (Alltech) in 30 minutes 0r 120 minutes respectively. To prevent unpacking, the pressure was bled from the bomb by slowly opening a prime/purge valve in-line with the pump. Columns were transported to a Varian HPLC pump, flushed with 90% acetonitrile at 4000 psi for 20 minutes and the pressure bled off by closing a prime/purge valve in-line with the pump. Flushing with acetonitrile resulted in packed beds being compacted by 2- 3 cm. The packed capillary

column is trimmed to the desired length (30-cm for L and 33 cm for D/L amino acid analysis) and threaded into the injection valve for use in separation.

APPENDIX B
ELECTRODE FABRICATION

Cylindrical carbon fiber microelectrodes (9 μm diameter, 1 mm in length) were used as working electrodes and were prepared in-house by previously described methods (Kawagoe et al., 1993; Knecht et al., 1984). A single carbon fiber (P-55's from Amoco Performance) is inserted into a borosilicate capillary (0.58 mm i.d., 1.0 mm o.d., 10 cm long) with filament by applying a small amount of suction at one end of the capillary. The glass capillary was pulled to a fine tip with a micropipette puller (PE-2 Narishige Scientific Instrument Lab, Tokyo Japan) to give 2 electrodes. The pulled ends of the electrodes were cut to a diameter of 15 – 20 μm using a disposable scalpel. The fiber was allowed to protrude about 0.5 mm from the glass by gently tapping the glass capillary. Carbon-fibers were sealed in the glass capillary by dipping in epoxy (Shell Epon Resin 828- 3.003 g heated to 80 °C and 0.4216 g metaphenylenediamine) for 20 seconds. Excess epoxy is removed from the exposed fiber by immediately dipping in hot acetone. The epoxy is allowed to gel overnight at room temperature and cured at 100 °C and 150 °C for 2 hours at each temperature. To make an electrical connection to the fiber, mercury was injected into the glass capillary with a needle, a wire inserted and sealed with super glue.

Reference electrodes were also prepared in-house. Silver wire (7 cm long) was first cleaned using 3 M HNO_3. Ag/AgCl electrodes were constructed by anodizing (at a current density of $0.4 mA cm^{-2}$) cleaned silver wire in 0.1 M HCl for 10 – 15 minutes or

until dark gray to black in appearance. The anodized wires were placed into tubes with semi-closed cracked tips containing saturated NaCl and solid NaCl and capped with rubber septa. Reference electrodes were stored in saturated NaCl solution for 1 week before testing. New electrodes were compared with previously made electrodes that were working well by placing in a NaCl solution and the voltage difference measured. If a potential difference of greater than 3mV was obtained, the new reference electrode was discarded.

APPENDIX C
USER PROGRAM

An automated instrument, Famos, was used to perform sample derivatization and injection. The Famos was used to produce more reproducible results over a long period of time due to the timing sequence which assures that samples are processed in exactly the same manner for exactly the same time. In this way, a large number of routine analyses were performed daily compared to manual timing and injection. The Famos used the above program to derivatize 2 μL of sample contained in a 250 μL tapered polypropylene microvial arranged in rows.

Reagent B was the transport liquid, same as mobile phase A, reagent C the OPA/thiol solution and reagent D, the IAA solution. For derivatization, 0.6 μL OPA/thiol was added (step 7) to the sample, mixed (steps 9 and 10) and allowed to react for 5 minutes. The excess thiol was removed by the addition of 0.4 μL IAA (step 16). After 3 minutes, a sandwich injection was be performed, 3 μL transport liquid, followed by 1 μL of sample, then 2.3 μL transport liquid (steps 23 – 31). An output signal was sent to the syringe pumps and the gradient started (step 32). The switching of the injection valve to the load position (step 34) was dependent on the time required for preconcentration (step 33). This time was set to preconcentrate up to 250 nL of sample at the head of the column. Wash steps were included throughout the program to clean the fused silica needle after exposure to different reagents and samples; the wash solution was composed of 20% propanol/water.

Steps 6, 11, 16, 20, 24, were included to ensure that there will be no air bubbles trapped in the fused silica needle as they could interfere with experimental reproducibility. The program was run in a loop format, i.e., derivatization of the next sample in the well plate was performed after an offset time has elapsed (step36). The steps of the user program are given in the table below.

FAMOS USER PROGRAM			
Step	**Vol (µL)**	**Command**	**Location**
1		Valve - load position	
2		Syringe valve- needle	
3		Wait 5 sec	
4	50	Wash	
5	2.0	Aspirate	Reagent C
6	1.0	Dispense	Waste
7	0.6	Dispense	Sample
8		Wait 5 sec	
9	1.8	Aspirate	Sample
10	1.8	Dispense	Sample
11	0.4	Dispense	Waste
12	50	Wash	
13		Wait 5 mins	
14	2.0	Aspirate	Reagent D
15	1.0	Dispense	Waste
16	0.4	Dispense	Sample
17		Wait 5 sec	
18	1.8	Aspirate	Sample
19	1.8	Dispense	Sample
20	0.6	Dispense	Waste
21	50	Wash	
22		Wait 3 mins	
23	5.0	Aspirate	Reagent B
24	2.0	Dispense	Waste
25	1.0	Aspirate	Sample
26		Wait 5 sec	
27	0	Aspirate	Sample
28	2.3	Aspirate	Reagent B
29		Wait 5 sec	
30	0	Aspirate	Reagent B
31		Valve position – inject	
32		Injection pulse marker on	
33		Wait 75 sec	
34		Valve position – load	
35	150	Wash	
36		Wait 10 mins	
37		End	

APPENDIX D
LC-SYSTEM TROUBLE SHOOTING GUIDE

Most of the work in this study was performed using the ISCO syringe pumps (operating at a flow rate of 40 μL/min) and the Famos Autosampler. The following section deals with some of the problems encountered with the pumps and the autosampler and ways to reduce, fix or work around the problem

Troubleshooting

Lubrication. Components of the Famos are not stainless steel, rusting results in friction along the guide arm. The arm requires cleaning bi-monthly with 20 % propanol and lubrication with WD-40 bi- monthly. Sign to look for before the onset of rusting includes squeaking noise during needle wash. This indicates there is a problem with the needle arm, lubricate and check for signs of rusting.

Air bubbles observed in chromatogram. This indicates a problem with the syringe or the fused silica needle. Air bubbles are often trapped within the syringe (and can be passed to the LC-system due to the connections to the valve) due to dissolved gasses in the wash solution or a defective syringe (usually if the plunger becomes separated from the Teflon tip). Wash solution must be thoroughly degassed, syringe must be removed and the air bubble removed. If the plunger has been separated from the Teflon, replacements (Teflon tips) are available from Dionex. If the fused silica needle outlet becomes chipped, the tip may not be reaching the bottom of the microvial. The syringe needle should be replaced or the volume scaled up to reduce the error in dispensing the

low volumes. Needles, if bought from Dionex can be expensive. However it is necessary to have a functional needle made by the manufacture to check the system.

Irreproducible Injections - May be due to incorrect volume added to the samples during derivatization or sample pick up volume incorrect. This may indicate a problem with the syringe, such as air bubbles in the syringe barrel. Irreproducibility may be as a result of leaking seals in the HPLC pumps. Procedure for seal replacement can be found in the ISCO manual. However, in the last few months, seal replacements were performed monthly. To ameliorate this problem, the pumps were operated at a higher flow rate (80 μL/min) and the splitter changed.

No injection on column - If the derivatization and sample pick up appear to be working properly, but no injection on column, this indicates a broken sample loop. In general, fused silica tubing should be replaced at regular intervals.

Error 75 – Error occurred during initialization, the FAMOS Well Plate cannot start. This error can also be caused by an incorrect plate format. The Famos is designed to be used with different well plates (48, 96, and 384). Check to see if the correct plate setting in the system menu is defined.

Error 40 – The sample needle spindle did not rotate correctly

Excess lubrication during maintenance can cause the spindle to 'over-rotate'. Any excess lubricant must be removed by blotting and the spindle adjusted manually by simply turning the spindle until it reaches half way. This must be done while the instrument is off. The needle arm must then be pulled slightly forward, but never pushed backward. When the instrument is turned on the arm should return to the default position.

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BIOGRAPHICAL SKETCH

Jacinth was born on July 2, 1972, in Montego Bay, Jamaica. She received her Bachelor of Science and her Master of Philosophy degrees in 1994 and 1997 respectively from the University of the West Indies, Mona, Jamaica. In August 1997, she enrolled at the University of Florida and begun further graduate studies under the direction of Dr. Robert T. Kennedy and was awarded a Doctor of Philosophy degree in August 2002.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert T. Kennedy, Chair
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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